

FORM PTO-1390

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371**

3428-005

U.S. APPLIC. NO. (if known, see 37 CFR 1.5)

08/973303

INTERNATIONAL APPLICATION NO

PCT/EP97/01590

INTERNATIONAL FILING DATE

March 27, 1997

PRIORITY DATE CLAIMED

March 28, 1996

TITLE OF INVENTION

PROTEIN WITH DIFFERENTIATION-INDUCING ACTIVITY FOR FRIEND'S ERYTHROLEUKEMIA CELL LINES

APPLICANT(S) FOR DO/EO/US

Peter DÖRMER

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1)
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau)
 - b. ☒ has been transmitted by the International Bureau
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2))
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau)
 - b. ☐ have been transmitted by the International Bureau
 - c. ☐ have not been made, however, the time limit for making such amendment has NOT expired
 - d. ☐ have not been made and will not be made
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4))
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5))

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
13. ☒ A **FIRST** preliminary amendment
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment
14. ☐ A substitute specification
15. ☐ A change of power of attorney and/or address letter
16. ☒ Other items or information
SEQUENCE LISTING
INTERNATIONAL SEARCH REPORT

U.S. APPLIC. NO (if known, see 37 CFR 1.50)	INTERNATIONAL APPLICATION NO PCT/EP97/01590	ATTORNEY'S DOCKET NUMBER 3428-005	
		CALCULATIONS	PTO USE ONLY
17. <input checked="" type="checkbox"/> The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO \$930.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) \$720.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) \$790.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$1070.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) \$98.00 <div style="text-align: right;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>		\$ 930.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e))		\$	
Claims	Number Filed	Number Extra	Rate
Total Claims	35 -20 =	15	x \$22.00
Independent Claims	5 -3 =	2	x \$82.00
Multiple dependent claim(s) (if applicable)			+ \$270.00
TOTAL OF ABOVE CALCULATIONS =			\$ 1424.00
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).			\$
SUBTOTAL =			\$ 1424.00
Processing fee of \$130.00 for furnishing the English translation later than the <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f))			+
TOTAL NATIONAL FEE =			\$ 1424.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) \$40.00 per property			+
TOTAL FEES ENCLOSED =			\$ 1464.00
Amount to be refunded			\$
charged			\$
a. <input type="checkbox"/> A check in the amount of \$ to cover the above fees is enclosed b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>12-2237</u> in the amount of \$ <u>1464.00</u> to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>12-2237</u> . A duplicate copy of this sheet is enclosed.			
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.			
SEND ALL CORRESPONDENCE TO <u>Frank P. Presta by Robert L. Prall, Reg.</u> <div style="display: flex; justify-content: space-between;"> <div> LOWE PRICE LEBLANC & BECKER 99 Canal Center Plaza, Suite 300 Alexandria, VA 22314 (703) 684-1111 </div> <div> SIGNATURE NAME 19,828 REGISTRATION NUMBER November 28, 1997 DATE </div> </div>			

Docket No.: 3373-042

PATENT

08/973303

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of
Gunilla JADESJÖ, et al
Serial No.
Filed: November 24, 1997
For: CHEMICAL COMPOSITION

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:
:PRELIMINARY AMENDMENT

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

IN THE SPECIFICATION

Please amend the title of the invention to read as follows
--Protein with Differentiation-Inducing Activity for Friend's
Erythroleukemia Cell Lines--.

Enclosed are copies of pages 39-47 of the published PCT
application which refer to the deposit made under the Budapest
Treaty. This deposit is State of the Art and was not made
together with the present application.

Enclosed with the application is the literal translation of
the claims as filed in the International Application.
Preliminary to examination of the above-referenced
application, please substitute the attached 7 sheets of claims
numbered pages 36-42 containing claims 1-35 for the English

translation of the claims as filed with the International Application. This is a page for page substitution resulting in the deletion of the multiple dependency of claims. Please further amend the attached substitute claims as follows:

IN THE CLAIMS

Page 36, please delete the title at the top of the page which reads "Novel protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines".

Claim 16, line 1, change "claims 13, 14, or 15" to --claim 13--.

Claim 17, lines 6 and 7, change "one or more of the preceding claims" to --claim 1--.

Claim 19, lines 1 and 2, change "one or more of the preceding claims" to --claim 1--.

Claim 21, line 2, delete " 14 or 15,".

Claim 23, lines 7 and 8, please change "one or more of the preceding claims" to --claim 1--.

Claim 27, lines 4 and 5, please change "one or more of the preceding claims" to --claim 1--.

Respectfully submitted,

LOWE PRICE LEBLANC & BECKER

Robert L. Price

Robert L. Price
Registration No. 22,685

99 Canal Center Plaza, Suite 300
Alexandria, Virginia 22314
(703) 684-1111 RLP:asg
Date: November 28, 1997

08³⁶/973303

Novel protein having at least differentiation-inducing
activity on Friend erythroleukemia cell lines

C L A I M S

1. Protein with at least differentiation-inducing activity on Friend erythroleukemia cell lines having the following properties wherein said protein has to comply with at least the features a), b), c), and d):
 - a) may be isolated from murine myelomonocytic leukemia cell lines;
 - b) may be isolated from irradiated human bone marrow stromal cell lines;
 - c) induces differentiation in Friend erythroleukemia cell lines with hemoglobin formation;
 - d) having a molecular weight in the range of about 10 - 60 kDa as determined by gel filtration on Sephacryl S300®;
 - e) with an expression of the corresponding mRNA in primary cells of the thymus, fetal liver, adult spleen, or bone marrow;
 - f) with characteristic repeat structures in the cDNA encoding the protein;
 - g) with corresponding mRNA species of different length consisting of identical 3' regions but different 5' regions.
2. Protein according to claim 1, characterized in that said protein has at least one of the following features:
 - h) showing a stable in vitro expression of the corresponding mRNA if an allogenic spleen cell reaction is carried out with non-irradiated, not pretreated spleen cells of mouse strains CBA and C57Bl/6;
 - i) having AT rich regions in the cDNA encoding the protein;
 - k) inducible by a serum factor present in fetal calf serum.
3. Protein according to claim 1, characterized in that

one or more of the repeat sequences presented in Table 3 or of repeat sequences hybridizing to these repeat sequences under stringent conditions are present in the DNA encoding the protein of claim 1 or claim 2.

4. Protein according to claim 1, characterized in that said protein may be isolated from human cells, murine cells, or the culture supernatants of human or murine cell lines.
5. Protein according to claim 1, characterized in that said protein exhibits a partial amino acid sequence encoded by a DNA hybridizing to the cDNA of SEQ ID NO:1 or NO:2 or NO:4.
6. Protein according to claim 5, characterized in that said protein exhibits a partial amino acid sequence encoded by a DNA hybridizing to the cDNA of SEQ ID NO:1 or NO:2 or NO:4 under stringent conditions.
7. Protein according to claim 1, characterized in that there are also comprised portions, analogues, and derivatives of said protein as well as fusion proteins each coding for a protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines.
8. Protein according to claim 1, having an essentially purified, native form.
9. Protein according to claim 1 having an essentially recombinant form.
10. Protein according to claim 1 said protein having at

least differentiation-inducing activity on Friend erythroleukemia cell lines and/or growth factor activity and/or colony-stimulating activity.

11. Protein according to claim 1, characterized in that said protein exhibits a differentiation-inducing effect on human leukemia cell lines.
12. Protein according to claim 1, characterized in that said protein contains partial amino acid sequences according to SEQ ID NO:3 or NO:5 wherein one or more of the amino acids may be deleted, substituted, or added each having at least differentiation-inducing activity on Friend erythroleukemia cell lines.
13. DNA fragment according to SEQ ID NO:1 or NO:2 or NO:4 or the complementary strand thereof, portions, derivatives, and analogues thereof each coding for a polypeptide having at least differentiation-inducing activity on Friend erythroleukemia cell lines.
14. DNA fragments, portions, analogues, and derivatives thereof each coding for a polypeptide having at least differentiation-inducing activity on Friend erythroleukemia cell lines hybridizing to the cDNA according to SEQ ID NO:1 or NO:2 or NO:4 and/or which are degenerated by the genetic code.
15. DNA fragments, portions, analogues, and derivatives thereof each coding for a polypeptide having at least differentiation-inducing activity on Friend erythroleukemia cell lines hybridizing to the cDNA according to SEQ ID NO:1 or NO:2 or NO:4 under stringent conditions and/or which are degenerated by the genetic code.

16. DNA fragment of claims 13, 14 or 15,
characterized in that
said DNA fragment encodes at least a part of a
polypeptide with the activity of the human or murine
protein having at least differentiation-inducing
activity on Friend erythroleukemia cell lines according
to one or more of the preceding claims.
17. Recombinant vector,
characterized in that
said vector contains a DNA sequence corresponding to a
gene or a DNA fragment encoding the protein with at
least differentiation-inducing activity on Friend
erythroleukemia cell lines according to one or more of
the preceding claims.
18. Recombinant vector according to claim 17,
characterized in that
said vector is derived from a bacterial plasmid, a
bacteriophage, or a viral vector.
19. Host cell transformed by a vector according to one or
more of the preceding claims.
20. Host cell according to claim 19,
characterized in that
said host cell is a prokaryotic cell or an eukaryotic
cell.
21. Method for the preparation of a DNA fragment according
to claim 13, 14 or 15,
characterized in that
said fragment comprises screening of a human or murine
cDNA clone library using as a probe a DNA fragment of a
DNA coding for a murine or human protein having at least
differentiation-inducing activity on Friend
erythroleukemia cell lines.

22. Monoclonal or polyclonal antibody directed against at least one epitope of a protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines according to claim 1.
23. Therapeutic, diagnostic or experimentally useful means, characterized in that
said means contains as an effective substance at least one nucleic acid in an effective amount which hybridizes to a gene or a part thereof encoding the protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines according to one or more of the preceding claims.
24. Means according to claim 23,
characterized in that
said means contains as an effective substance at least one nucleic acid comprising (a) the nucleotide sequence encoding a protein with at least differentiation-inducing activity on Friend erythroleukemia cell lines, (b) a portion thereof, (c) a nucleotide sequence hybridizing to a nucleic acid as under (a) and/or (b) under stringent conditions, or (d) a nucleotide sequence complementary to a nucleotide sequence as under (a), (b), and/or (c).
25. Means according to claim 23,
characterized in that
said nucleic acid optionally is a modified DNA.
26. Means according to claim 23,
characterized in that
said nucleic acid optionally is a modified RNA.
27. Therapeutic means,
characterized in that
said means contains a protein, an analogue, a derivative or portions thereof according to one or more of the

- preceding claims each functioning as a polypeptide with at least differentiation-inducing activity on Friend erythroleukemia cell lines together with conventional carriers and excipients in an effective amount.
28. A molecular probe in diagnostics or therapy comprising a means according to claim 23.
 29. An antisense nucleic acid for the inhibition of gene expression comprising a means according to claim 23.
 30. DNA encoding a protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines, a portion, derivative, or analogue thereof each functioning as a polypeptide with at least differentiation-inducing activity on Friend erythroleukemia cell lines for the incorporation into a prokaryotic or eukaryotic cell.
 31. Fusion protein having an amino acid sequence consisting completely or in part of the amino acid sequence of the human or murine protein with at least differentiation-inducing activity on Friend erythroleukemia cell lines according to claim 1 and in part of a prokaryotic and/or eukaryotic protein.
 32. Synthetic protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines according to claim 1 and having an amino acid sequence at least part of which is encoded by a DNA sequence hybridizing to the DNA sequence according to SEQ ID NO:1 or NO:2 or NO:4 at least under stringent conditions.
 33. A protein according to claim 1 or inhibitors of said protein for the treatment of diseases in which a local or systemic overproduction or underproduction of this protein affects the development of the disease or the course thereof.

34. A protein according to claim 1 as a growth factor, colony-stimulating factor, a factor inducing erythropoiesis and/or inducing the immune system.
35. Protein according to claim 1, characterized in that said protein comprises at least those amino acids which are encoded by nucleotides 74 - 154 or 155 - 685.

.HEADLINE

ATCC CCL-243

K-562 (Chronic myelogenous leukemia, human)

.TEXT

Current medium for propagation: RPMI 1640, 90%; FBS, 10%.
 The continuous cell line K-562 was established by Lozzio and Lozzio (Blood 45: 321-334, 1975) from the pleural effusion of a 53-year-old female with chronic myelogenous leukemia in terminal blast crises. The cell population has been characterized as highly undifferentiated and of the granulocytic series (Leukemia Res. 3: 363-370, 1979). Studies conducted by Anderson, et al., (Int. J. Cancer 23: 143-147, 1979) on the surface membrane properties led to the conclusion that the K-562 was a human erythroleukemia line. The effect of inducers on sublines derived from the original K-562 cell line have been reviewed by Koeffler and Golde (Blood 56: 344-350, 1980). More recent studies indicate that the K-562 blasts are multipotential, hematopoietic malignant cells that spontaneously differentiate into recognizable progenitors of the erythrocytic, granulocytic and monocytic series (Proc. Soc. Exp. Biol. Med. 166: 546-550, 1981).
 The K-562 cell line has attained widespread use as a highly sensitive in vitro target for the natural killer assay (J. Natl. Cancer Inst. (Bethesda) 59: 77-83, 1977). Cultures from the ATCC stock have been shown to exhibit this sensitivity for assessing human natural killer activity.

Karyological studies on various K-562 sublines have been classified into three groups (A,B,C) by Dimery, et al., (Exp. Hematol. 11: 601-610, 1983). The strain obtained by the ATCC most closely resembles the B population. Occurrence of the Philadelphia chromosome, however, was of much lower frequency; none detected in 15 metaphases examined.

DESCRIPTION OF REPOSITORY REFERENCE SEED STOCK

Number of Serial Subcultures from Tissue of Origin: Unknown.

Freeze Medium: Culture medium, 95%; dimethyl sulfoxide (DMSO), 5; antibiotic-free.

Viability: Approximately 85% (dye exclusion).

Culture Medium: RPMI medium 1640, 90%; FBS, 10%; antibiotic-free.

Growth Characteristics of Thawed Cells: An inoculum of 1-2 X 10(5) viable cells/ml in the above culture medium at 37C results in a doubling time of 26-30 hrs over a 5- to 7-day period provided fresh medium is added at 48-72 hr intervals.

Plating Efficiency: The cells cannot be plated.

Morphology: Lymphoblast-like.

Karyology: Chromosome Frequency Distribution 50 Cells: 2n = 46
 cells: 1 1 10 12 7 13 3 2 1

Chromosomes: 64 65 66 67 68 69 70 72 77

The stemline chromosome number is triploid with the 2S component occurring at 4.2%. Fifteen markers (M1 and M(15)) occurred in nearly all S metaphases. Spontaneous non-specific dicentrics occurred, but rarely. Unstable markers were also rarely seen. The X was disomic, and N9 was nullisomic.

Sterility: Tests for mycoplasma, bacteria, fungi, protozoa and viruses were negative.

Species: Confirmed as human by isoenzymology.

Tumorigenicity: Tumors developed in 30-35 days at 33 1/3% frequency (2/6) in nude mice inoculated subcutaneously with 10(7) cells.

EBNA: Negative.

Reverse Transcriptase: Not detected.

Erythrocyte Rosette Test: E, 1%; EA, 34%; EAC, 2%.

HLA Profile: Not detected.

Isoenzymes: AK1, 1; ES D, 1; GLO-1, 2; G6PD, B; PGM1, 0; PGM3, 1; Me-2, 0.

Submitted by: H.T. Holden, NCI, NIH, Bethesda, MD.

Prepared and characterized by: ATCC, Rockville, MD.

Price Code: J

.HEADLINE

ATCC TIB-68

WEHI-3 (Myelomonocyte, mouse)

.TEXT

Passage Frozen: Unknown. Current medium for propagation: Iscove's modified Dulbecco's medium with 2-mercaptoethanol, $10(-5)M$, 90%; FBS, 10%. Additional Information: This macrophage-like line was derived from a BALB/c mouse. Growth of these myelomonocytic leukemia cells is inhibited by concentrations of LPS as low as 4.0 ng/ml and blocked completely at higher concentrations. Dextran sulfate also inhibits growth at concentrations of 30-40 mcg/ml. Production of the constitutive enzyme lysozyme and of granulocyte colony-stimulating activity (CSA) by WEHI-3 is unaffected or actually enhanced during inhibition of cell growth. Latex beads are phagocytized but not toxic. Zymosan and BCG are also phagocytized and block growth. The cell surface bears receptors for immunoglobulin and complement. WEHI-3 exhibits only weak effector activity against sheep erythrocytes or the tumor target EL-4 in an antibody-dependent cell mediated cytotoxic system. References: Cancer Res. 37: 546-550, 1977; J. Immunol. 119: 950-954, 1977; J. Exp. Med. 143: 1528-1533, 1976; ibid., 154: 1419-1431, 1981. Submitted by: Laboratory of M. Cohn, Salk Institute, La Jolla, CA.
Price Code: J

DUPLIKAT

BUDAPESTER VERTRAG ÜBER DIE INTERNATIONALE
ANERKENNUNG DER HINTERLEGUNG VON MIKROORGANISMEN
FÜR DIE ZWECKE VON PATENTVERFAHREN

INTERNATIONALES FORMBLATT

GSF-Institut für
Experimentelle Hämatologie
Marchioninstr. 25
8000 München 70

DSMZ Deutsche Sammlung von Mikroorganismen

und Zellkulturen GmbH
11.02.92

LEBENSFÄHIGKEITSBESCHEINIGUNG
ausgestellt gemäß Regel 10.2 von der unten angegebenen
INTERNATIONALEN HINTERLEGUNGSSTELLE

I. HINTERLEGER	II. KENNZEICHNUNG DES MIKROORGANISMUS
Name: GSF-Institut für Experimentelle Hämatologie Adresse: Marchioninstr. 25 8000 München 70	Von der INTERNATIONALEN HINTERLEGUNGSSTELLE zugewiesene EINGANGSNUMMER: DSM ACC2056 Datum der Hinterlegung oder Weiterleitung ¹ : 1993-01-27
III. LEBENSFÄHIGKEITSBESCHEINIGUNG	
Die Lebensfähigkeit des unter II genannten Mikroorganismus ist am 1993-01-27 ² geprüft worden. Zu diesem Zeitpunkt war der Mikroorganismus <input checked="" type="checkbox"/> ³ lebensfähig <input type="checkbox"/> ³ nicht mehr lebensfähig	
IV. BEDINGUNGEN, UNTER DENEN DIE LEBENSFÄHIGKEITSPRÜFUNG DURCHGEFÜHRT WORDEN IST ⁴	
IV. INTERNATIONALE HINTERLEGUNGSSTELLE	
Name: DSM DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Anschrift: Mascheroder Weg 1 B D-3300 Braunschweig	Unterschrift(en) der zur Vertretung der internationalen Hinterlegungsstelle befugten Person(en) oder des (der) von ihr ermächtigten Bediensteten: <i>Detmar Tüke</i> Datum: 1993-02-12

¹ Angabe des Datums der Ersthinterlegung. Wenn eine erneute Hinterlegung oder eine Weiterleitung vorgenommen worden ist, Angabe des Datums der jeweils letzten erneuten Hinterlegung oder Weiterleitung.

² In den in Regel 10.2 Buchstabe a Ziffer ii und in vorgesehenen Fällen Angabe der letzten Lebensfähigkeitsprüfung.

³ Zutreffendes ankreuzen.

⁴ Ausfüllen, wenn die Angaben beantragt worden sind und wenn die Ergebnisse der Prüfung negativ waren

DUPLIKAT

BUDAPESTER VERTRAG ÜBER DIE INTERNATIONALE
ANERKENNUNG DER HINTERLEGUNG VON MIKROORGANISMEN
FÜR DIE ZWECKE VON PATENTVERFAHREN

INTERNATIONALES FORMBLATT

DSMZ-Deutsche Sammlung von Mikroorganismen
und Zellkulturen GmbH
U. W. 11.02.97

GSF-Institut für
Experimentelle Hämatologie
Marchioninstr. 25
8000 München 70

EMPFANGSBESTÄTIGUNG BEI ERSTHINTERLEGUNG,
ausgestellt gemäß Regel 7.1 von der unten angegebenen
INTERNATIONALEN HINTERLEGUNGSSTELLE

I. KENNZEICHNUNG DES MIKROORGANISMUS	
Vom HINTERLEGER zugeordnetes Bezugszeichen: L8815	Von der INTERNATIONALEN HINTERLEGUNGSSTELLE zugeordnete EINGANGSNUMMER: DSM ACC2056
II. WISSENSCHAFTLICHE BESCHREIBUNG UND/ODER VORGESCHLAGENE TAXONOMISCHE BEZEICHNUNG	
<p>Mit dem unter I. bezeichneten Mikroorganismus wurde</p> <p>() eine wissenschaftliche Beschreibung</p> <p>(X) eine vorgeschlagene taxonomische Bezeichnung</p> <p>eingereicht. (Zutreffendes ankreuzen).</p>	
III. EINGANG UND ANNAHME	
<p>Diese internationale Hinterlegungsstelle nimmt den unter I. bezeichneten Mikroorganismus an, der bei ihr am 1993-01-27 (Datum der Ersthinterlegung)¹ eingegangen ist.</p>	
IV. EINGANG DES ANTRAGS AUF UMWANDLUNG	
<p>Der unter I. bezeichnete Mikroorganismus ist bei dieser internationalen Hinterlegungsstelle am eingegangen (Datum der Ersthinterlegung) und ein Antrag auf Umwandlung dieser Ersthinterlegung in eine Hinterlegung gemäß Budapester Vertrag ist am eingegangen (Datum des Eingangs des Antrags auf Umwandlung).</p>	
V. INTERNATIONALE HINTERLEGUNGSSTELLE	
<p>Name: DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH</p> <p>Adresse: Mascheroder Weg 1 B D-3300 Braunschweig</p>	<p>Unterschrift(en) der zur Vertretung der internationalen Hinterlegungsstelle befugten Person(en) oder des (der) von ihr ermächtigten Bediensteten:</p> <p><i>Daymar Tula</i></p> <p>Datum: 1993-02-12</p>

¹ Falls Regel 6.4 Buchstabe d zutrifft, ist dies der Zeitpunkt, zu dem der Status einer internationalen Hinterlegungsstelle erworben worden ist.
Formblatt DSM-BP/4 (einige Seite) 0221

Literatur

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Novel protein having at least differentiation-inducing
activity on Friend erythroleukemia cell lines

The invention is directed to a novel protein with differentiation-inducing activity which may be isolated from mammalian cells and in particular from culture supernatant of mammalian cell cultures. In particular, the protein may be isolated from murine and human cells.

It is an object of the present invention to provide a novel protein having at least differentiation-inducing activity, particularly on Friend's erythroleukemia cell lines.

According to the invention, this object has been achieved by providing a protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines and having at least the features a), b), c), and d):

- a) may be isolated from murine myelomonocytic leukemia cell lines;
- b) may be isolated from irradiated human bone marrow stromal cell lines;
- c) induces differentiation in Friend erythroleukemia cell lines with hemoglobin formation;
- d) having a molecular weight in the range of about 10 - 60 kDa as determined by gel filtration on Sephacryl S300®;
- e) with an expression of the corresponding mRNA in primary cells of the thymus, fetal liver, adult spleen, or bone marrow;
- f) with characteristic repeat structures in the cDNA encoding the protein;
- g) with corresponding mRNA species of different length consisting of identical 3' regions but different 5' regions.

Preferably, the protein provided by the invention additionally shows at least one of the following features:

- h) showing a stable in vitro expression of the corresponding mRNA if an allogenic spleen cell reaction is carried out with non-irradiated, not pretreated

spleen cells of mouse strains CBA and C57Bl/6;

- i) inducible by a serum factor present in fetal calf serum;
- k) having AT rich regions in the cDNA encoding the protein.

An activity has been discovered in the culture supernatant of a murine myelomonocytic leukemia cell line (WEHI-3) which induces murine erythroid cells (Friend erythroleukemia cell lines) to differentiate under concurrent hemoglobin formation. The molecular weight of this activity is between about 10 and 60 kDa with protein species or protein aggregates of different sizes assumed. Expression of the activity is dependent on a serum factor which so far has not been characterized and which is present in varying concentrations in different batches of commercially available fetal calf serum. An identical activity with respect to its effect has been discovered also in the supernatant of irradiated human bone marrow stromal cells (cell line L88/5). The activity has been preliminarily designated by EDA (erythroid differentiation activity). In the further course of the investigation also other than erythropoiesis-inducing functions have been discovered (see below). In addition, EDA has been demonstrated to have an erythropoiesis-inducing effect on human leukemia cell line K562 (species-crossing effect of EDA).

A small cDNA species of the gene (DY-8) was found by expression cloning which after transfection into Cos-1 cells resulted in a culture supernatant with EDA activity. This 715 bp cDNA was used as a probe for the isolation of a larger cDNA fragment of about 1,350 bp (HA-15/2) which after transient expression in Cos-1 cells also exhibited a weak EDA effect if the Cos-1 supernatant was used. In the murine cells examined, the gene is expressed in the form of different RNA species (presumably splice variants) of about 800, 1,200, 1,350, 1,750, and 2,200 bp. Expression of the *eda* mRNA, in some cases to a very low extent, was detectable in all tissues examined (liver, kidney, brain, intestine, placenta). The highest expression was detected in the thymus, followed by fetal liver, adult spleen, and bone marrow. Many hematopoietic mouse cell lines, especially those with

leukemic transformation such as DA-3, WEHI-3, NFS-60, or NFS-61, but also those without leukemic transformation such as NIH-3T3 or TS1-C3, tend to show very high eda expression at the RNA level.

Using the murine eda probe, HA-15/2, we were able to detect a corresponding human gene at the DNA level by Southern blot analysis under stringent conditions as well as at the RNA level using Northern blot analysis. The Jurkat cell line was particularly positive in this respect. In addition, a distinct band of about 1,100 bp was found in a case of human chronic T cell leukemia of the T helper cell type which was not detected in any other of the 8 samples of human cell lines and primary human bone marrow material.

In all of the experiments conducted, the 2.2 kbp band of the eda mRNA showed the most consistent expression pattern. Its structure as analysed in NIH-3T3 cells is depicted in Fig. 19. The repeat structures represent an important characteristic of the eda cDNA (Table 3). The corresponding sequence, a consensus sequence derived from clones of WEHI-3 and NIH-3T3 cell lines, is presented in Fig. 18 which corresponds to SEQ ID NO:1. All mRNA species, i.e. the bands of 800, 1,200, 1,350, 1,750, and 2,200 bp, have an identical 3' end designated by "tail" in Fig. 19 while it is still unclear whether in each case there is a band-specific 5' end or the bands represent different splicing products. Since the differentiation-inducing effect on erythropoietic cells was discovered using clone DY-8 which contains about 640 bp of the 3' region of eda together with 73 bp of its specific 5' end (Fig. 20 includes SEQ ID NO:2) the differentiation-inducing function is essentially associated with the 3' end of the cDNA.

A comparison of the band sizes of the various eda mRNA species in different mouse strains such as Balb/c, C3H, CBA, C57B16, Swiss, AKR, or NFS, as well as of partial sequences of the eda cDNA of different mouse strains reveal a mouse strain-dependent variability which is the result of different frequencies of the various repeats in individual mouse strains. Because of the high eda expression in some murine

tumor cell lines a role in tumor cell growth may be expected.

Since the 5' end of the open reading frame has still not been unambiguously defined, also a second protein SEQ ID NO:5 of 206 amino acids starting further upstream from the 5' end of the 177 amino acid protein SEQ ID NO:3 which was found by expression cloning was permanently expressed in a human colon carcinoma cell line CX2. This larger protein has a differentiation-inducing activity towards murine Friend erythroleukemia cells which is 1 - 2 dilution grades higher.

Although eda is expressed in many cell lines, the EDA activity is found in the culture supernatants of only a small number of cell lines. It is detectable if the cells are irradiated or if a major protein depletion takes places, i.e. under stress conditions.

The study of the role of eda in normal spleen cells of the mouse revealed that a mitogenic or T cell receptor-specific stimulation of the cells or a stimulation of protein kinase C leads to the rapid disappearance of the various mRNA species which is accompanied by degradation products of defined size. In contrast, a stable expression occurs if an allogeneic spleen cell reaction is carried out with non-irradiated and non-pretreated spleen cells of mouse strains CBA and C57Bl/6. From these results a significance of the involvement in the allogeneic spleen cell reaction may be concluded which was emphasized by a semi-allogeneic mouse transplantation model (C57Bl/6 spleen cells injected into lethally irradiated CBAxC57Bl/6 mice). In the spleens of the test animals an about 7-fold increase in eda expression occurred in the course of an acute "graft-versus-host" disease as compared to the controls which had undergone compatible tissue transplantation.

Already very low concentrations of the recombinant supernatants of the CX2 cell line are able to stimulate the BL-70 Burkitt's lymphoma cell line in the sense of a growth factor. In addition, also recombinant supernatants of cell line CX2 have a colony-stimulating effect on human hematopoietic progenitors in the fraction of CD34+ bone marrow cells.

The protein provided by the invention may be isolated from mammalian cells, particularly from the supernatant of mammalian cell cultures. In a preferred embodiment of the invention, it is isolated from murine or human cells. Preferred cell lines include for example: the murine WEHI-3 myelomonocytic leukemia cell line, ATCC TIB68, and the irradiated L8815 human bone marrow stromal cell line, DSM ACC 2056. In the following, the L8815 cell line will be designated by L88/5. Friend erythroleukemia cell lines F4N or B8/3 were used for detection of the protein.

In the present application, the individual cell lines from which the inventive protein provided may be isolated and on which the effects mentioned may be demonstrated are only presented by way of example. The skilled expert will be able to practise the invention also using cell lines different from those mentioned above. Already for this reason, a deposition of these cell lines becomes unnecessary. Furthermore, in the references cited in the list of references the cell lines are described in a manner sufficient and thus reproducible. It should again be noted that for the practice of the present invention it is not required to use the cell lines described but that also other cell lines may be used which can be determined by the skilled expert by means of routine experimentation.

For WEHI-3, a computer printout from the ATCC catalogue has been included; the same has been done for cell line K562. Reference is made to the references cited therein in their entirety.

The protein provided according to the invention includes a partial amino acid sequence encoded by a DNA hybridizing to the cDNA of SEQ ID NO:1 or NO:2 or NO:4. The hybridization is preferably carried out under stringent conditions. The protein contains at least those amino acids encoded by the nucleotide sequence which has been referred to as "consensus sequence" in Fig. 20. Deletions, insertions, amino acid exchanges, and amino acid modifications are possible inasmuch as they do not interfere with protein function.

Stringent conditions in the sense of the present

invention are those conditions enabling selective and detectable specific binding of the nucleic acid to the gene coding for the protein of the invention or to transcripts of the gene coding for the protein of the invention. A hybridization of this type under stringent condition is preferably meant to be a hybridization at 65°C in an aqueous solution or at 42°C in 50% formamide and subsequent washing of the filter at 60°C in an aqueous solution having a salt concentration of 15 mM NaCl and a concentration of SDS of 0.1% after which binding of the probe to the gene coding for the protein of the invention or to a RNA derived therefrom can be detected. If shorter nucleic acids are used as probes it may be necessary to employ less drastic hybridization and/or washing conditions.

The present invention is meant to comprise also portions, analogues, and derivatives of the protein of the invention as well as fusion proteins. The protein according to the invention preferably is of essentially purified and native form or of essentially recombinant form and exhibits at least differentiation-inducing activity on Friend erythroleukemia cell lines.

The protein of the invention shows differentiation-inducing activity. This activity has been demonstrated for murine Friend virus-transformed erythroleukemia cell lines as well as for a human leukemia cell line, i.e. for K562. It may be expected that a similar differentiation-inducing effect on other related cell lines and especially on human leukemia cell lines will be detected in addition. Moreover, it may be expected because of the present results of the investigation that this differentiation-inducing activity is effective not only on erythropoietic cells but also on other cells. Furthermore, it exhibits a growth factor effect as well as a colony-stimulating effect.

To characterize the protein, the specification as well as the claims always refer exemplarily to the differentiation-inducing activity towards Friend erythroleukemia cell lines. Further activities which have already been detected according to the invention are

presented in the present specification. It may be expected that the protein provided herein exhibits also other activities which are inherent to the protein. Thus, the activities mentioned contribute only to a detailed characterization and distinction of the protein and are not intended to represent a conclusive list of its features, properties, and activities.

Thus, it has been found according to the invention that the protein provided has an erythropoiesis-inducing effect on human leukemia cell lines, for example on K 562 (ATCC No. CRL243).

The present invention comprises DNA fragments according to SEQ ID NO:1 or NO:2 or NO:4, portions, derivatives, and analogues thereof, each encoding a polypeptide having at least differentiation-inducing activity on Friend erythroleukemia cell lines and which hybridize to the cDNA of SEQ ID NO:1 or NO:2 or NO:4, preferably under stringent conditions.

The present invention is also directed to DNA fragments encoding at least part of a polypeptide which has the activity of the human or murine protein with at least differentiation-inducing activity for example on Friend erythroleukemia cell lines according to the present invention.

Furthermore, the present invention is directed to recombinant vectors containing a DNA sequence corresponding to a gene or a DNA fragment coding for a protein with differentiation-inducing activity on Friend erythroleukemia cell lines according to the invention. The vectors of the invention may represent vectors according to the state of the art, for example bacterial plasmids or viral vectors. Also comprised are expression vectors.

Further, the invention comprises host cells transformed by a vector provided by the invention. The host cells may be prokaryotic or eukaryotic cells, for example E.coli cells or yeast cells.

The DNA fragments according to SEQ ID NO:1 or NO:2 or NO:4 of the invention, portions, derivatives, or analogues

thereof each coding for a polypeptide having at least differentiation-inducing activity on Friend erythroleukemia cell lines may be prepared according to the invention for example by screening a human or murine cDNA clone library using a DNA fragment of a DNA coding for a murine or human protein with differentiation-inducing activity.

The present invention further relates to monoclonal or polyclonal antibodies directed against at least one epitope of a protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines.

In addition, according to the invention there is provided a therapeutic, diagnostic, or experimentally useful means containing as an active ingredient at least one nucleic acid in an effective amount which hybridizes to a gene or a portion thereof, and encoding the protein of the invention which has at least differentiation-inducing activity on Friend erythroleukemia cell lines.

Also, there is provided according to the invention a therapeutic, diagnostic, or experimentally useful means characterized in that said means contains as an active ingredient at least one nucleic acid comprising (a) the nucleotide sequence encoding a protein with at least differentiation-inducing activity on Friend erythroleukemia cell lines, (b) a portion thereof, (c) a nucleotide sequence hybridizing to a nucleic acid as under (a) and/or (b) under stringent conditions, or (d) a nucleotide sequence complementary to a nucleotide sequence as under (a), (b), and/or (c). The nucleic acid of said means optionally may be a modified DNA or RNA.

The therapeutic means provided according to the invention includes the protein having at least differentiation-inducing activity for example on Friend erythroleukemia cell lines of the present invention, an analogue, derivative, or portions thereof, each together with conventional carriers and/or adjuvants in an effective amount.

The therapeutic, diagnostic, or experimentally useful means according to the invention may for example be used as a

molecular probe in diagnostics or as an antisense nucleic acid for the inhibition of gene expression. By use of antibodies against this means, the differentiation-inducing effect may be therapeutically, diagnostically, or experimentally modulated.

Furthermore, the invention relates to a method for the transformation of a prokaryotic or eukaryotic cell using a DNA which encodes the protein provided according to the invention having at least differentiation-inducing activity on Friend erythroleukemia cell lines-as well as portions, derivatives, or analogues of this DNA having said activity.

Moreover, the invention is directed to a fusion protein having an amino acid sequence consisting completely or in part of the amino acid sequence of the human or murine protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines according to the invention and in part of a prokaryotic or eukaryotic protein.

Further, the invention is directed to a synthetic protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines according to the invention which has an amino acid sequence encoded by a DNA sequence hybridizing to the DNA sequence according to SEQ ID NO:1 or NO:2 at least under stringent conditions.

The protein provided according to the invention is preferably useful for the treatment of diseases accompanied by impairments of the differentiation-inducing activity in erythropoietic cells.

Fields of the invention :

Further, it is preferably suitable for the treatment of diseases wherein the formation of blood cells in bone marrow and/or lymphopoietic tissue is impaired owing to illness or treatment.

Furthermore, it is preferably suitable for the treatment of cells of blood-forming tissues within or outside the organism for the purpose of achieving a proliferation of precursor cells and/or stem cells of blood formation with or without a genetic alteration.

Moreover, it is preferably suitable for the modulation of immunological processes wherein cellular recognition or cellular elimination processes are supposed to take place in the sense of a mixed spleen cell reaction.

In the following, the invention will be explained in more detail with respect to the accompanying Figures and regarding preferred embodiments. However, the invention is not restricted to the preferred embodiments described.

The Figures show :

Fig. 1 : Induction of erythroid differentiation in murine F4N erythroleukemia cells by 4 different WEHI-3-conditioned media

Fig. 2 : Induction of erythroid differentiation in murine B 8/3 erythroleukemia cells by culture supernatants of the human stromal cell line L88/5 measured on days 3 and 4

Fig. 3 : Induction of erythroid differentiation in human K562 CML cells by 4 different WEHI-3-conditioned media. In particular, an effect is recognizable with WCM (C)

Fig. 4 : Effect of WEHI-3-conditioned medium on the cell number and the α -globin synthesis of B 8/3 mouse erythroleukemia cells

Fig. 5 : Effect of WEHI-3-conditioned medium on the adherence of WEHI-3 cells to the plastic bottoms of culture flasks, the culture period being 72 hours. The relative cell number was measured by the MTT test.

Fig. 6 : Gel chromatographic fractionation of WEHI-3-conditioned medium using Sephacryl S 300®. Biological testing of the fractions for EDA activity was performed using B 8/3 mouse erythroleukemia cells, the culture period being 4 days. The induction of α -globin mRNA was measured using Northern blot analysis

Fig. 7 : Dependence of EDA production on the density of WEHI-3 cells on harvesting the cells from the FCS-containing primary culture. Activity was determined by culturing B 8/3 mouse erythroleukemia cells with WEHI-3 supernatants for 3 days and counting the percentage of benzidine-positive cells

Fig. 8 : Fractionation of the supernatant of experiment W3/2 (see Fig. 7) and testing the fractions in culture with B 8/3 mouse erythroleukemia cells and counting the percentage of benzidine-positive cells

Fig. 9 : Steps of expression cloning of a murine eda sequence by stepwise reduction of the number of clones of an expression library which were included in the biological testing

Fig. 10 : Induction of murine B 8/3 erythroleukemia cell differentiation by Cos-1-conditioned supernatants in 3 independent experiments following transfection with clone DY-8 or an irrelevant control clone BY-7, respectively. For comparison, WEHI-3 cell supernatant was included in the testing as a positive standard. Mean values \pm 1 standard deviation of individual results from the BY-7 control clone are represented as hatched area. Additionally, for comparison purposes \pm 1 standard deviation of the results obtained with DY-8 is shown as bars.

Fig. 11 : Expression of the truncated eda clones HA-15/2 and HA-12/1 in Cos-1 cells and testing of the Cos-1 supernatants in B 8/3 mouse erythroleukemia cells in culture samples over a period of 3 and 4 days. The percentage of benzidine-positive cells was evaluated. On day 4, the difference between the control and the result obtained with clone HA-15/2 is hardly above the 5% significance limit

Fig. 12 : Southern blot analysis of different murine DNAs cut with EcoRI using the murine HA-15/2 eda probe. Also analysed was the DNA of the human K562 cell line (4th lane

from the left) showing a weak positive signal of bands of 7.5 and 6.5 kbp upon highly stringent washing

Fig. 13 : Expression of *eda* in the RNA of murine NIH-3T3 (always on the left) and M2-10B4 (always on the right) cell lines. Northern blot hybridization was carried out with different probes as detailed in the text

Fig. 14 : Northern blot analysis of the RNA of various human cell lines using the murine HA-15/2-derived *eda* probe. For each of the cell lines H33, Reh, and K562 15 μ g of total RNA was used; for the Jurkat sample about 5 μ g of poly(A)+ RNA was used

Fig. 15 : Expression of *eda* in murine spleen cells (strain C3H) after stimulation with anti-T cell receptor antibody or concanavalin A. The densitometry results of the Northern blot analysis after hybridization with the murine HA-15/2-derived *eda* probe are presented in separate for the large *eda* bands of 2,200 and 7,000 bp and for the smaller bands of 600 and 400 bp presumably representing degradation products

Fig. 16: Expression of *eda* in murine spleen cells of mouse strains CBA and CBL upon performing a 3-day mixed spleen cell culture. Expression of *eda* in murine placenta and murine fetal liver (d 15) are shown as controls. Northern blot analysis with 5 - 15 μ g of total RNA using *eda* clone HA-15/2 as a probe. The amount of RNA loaded to the individual lanes may be seen from the subsequent hybridization of the RNA samples of the same filter to a 28 S RNA probe

Fig. 17 : Expression of the 2.2 kbp *eda* mRNA species in a semi-allogenic transplantation (CBL cells in (CBA x CBL) F_1 hybrids) or an autologous transplantation, respectively, of CBA x CBL spleen cells into (CBA x CBL) F_1 hybrids. Transplantation was carried out with 5×10^7 spleen cells per animal. Expression of *eda* in CBL cells on day 0 was taken as

100%. Northern blot analysis with 15 μ g of total RNA per each lane using the murine HA-15/2-derived eda probe

Fig. 18 : Consensus partial sequence of the 2,200 bp eda cDNA. This sequence lacks a continuous open reading frame, with possibly individual fragments missing from the sequence

Fig. 19 : Preliminary structure of the 2.2 kbp eda cDNA. Abbreviations : R 1-1 to R 1-3 = 81 bp repeats No. 1-3; R 2-1 and R 2-2 = approx. 180 bp repeats Nos. 1 and 2; Tail = 3' end of the gene which is identical for all mRNA species; Unknown = approx. 300 bp of up to now unknown sequence; Start? = 81 bp fragment from the 5' region the exact localization of which within the sequence at the beginning of the 2.2 kbp eda cDNA still has to be determined. The open reading frame found by expression cloning resides in the black part of the region designated by "Tail" wherein a 385 bp repeat (R 3-1) was present in only 2 of 32 clones which is presumably spliced out prior to translation to remove the stop codon and the "frameshift"

Fig. 20 : Sequence of clone DY-8 consisting of 715 bp. The first 73 bp (in italics) determine the specific 5' end of this clone. starting with position 74, the clone largely corresponds to the 3' region of the presumptive 2.2 kbp eda species as indicated by the term "consensus sequence". The sequence coding for 177 amino acids starts with the boxed ATG at position 155 and is printed in bold letters. An almost identical sequence of 636 bp is presented in the lower row into which an ATG was artificially inserted at position 52 leading to an open reading frame for 206 amino acids

Fig. 21 : Differentiation-inducing effect of various conditioned media on murine erythroleukemia cell line B 8/3. The values obtained from the control media (+/- 1 standard deviation) are represented by a grey bar. The CX-2 control supernatant after transformation with vector alone is referred to as "CX-2 + Rc/CMV". Standard WCM (WEHI-3 CM) was

used as a positive control

Fig. 22 : Effect of different concentrations of the supernatant of cloned CX2 cell line transfected by the 534 bp eda in vector Rc/CMV (CX2-C9) on the growth of cells of the BL-70 Burkitt's lymphoma cell line. OD measurements were performed in microwells using the MTT test after 4 days of culture. A : Dependence of growth on seeding cell concentration, in the presence or absence of different concentrations of CX2 supernatant. B : Relationship between the growth of BL-70 cells and the concentration of recombinant CX2 supernatant added for 3 different cell densities in the same experiment

Fig. 23 : Methyl cellulose cultures of human CD34+ bone marrow progenitors with and without addition of eda in two different constructs. A : Number of mixed colonies of a normal donor after 20 days of culture with and without addition of 10% of the supernatant of bladder carcinoma cell line 5637. Two different supernatant concentrations (5 and 17%) of transfected but not cloned CX2 cells were used. * = $p < 0.05$; ** = $p < 0.01$. B : Supernatant from a CX2 cell line was used which was cloned and transfected with 534 bp eda in Rc/CMV vector (CX2-C9). Colony counts after 14 days of BFU-E and GM-CFC are shown from a normal bone marrow donor (upper part) and a CML patient (lower part).

The accompanying Tables show :

Table 1 : Percentage of reduction in EDA activity

Table 2 : Cytokins without differentiation-inducing
activity on murine erythroleukemia cells

Table 3 : Repeat structures in the presumptive consensus
partial sequence of the 2,200 bp eda DNA.

Materials and Methods

Cell lines :

Experiments were performed using the following cell lines: murine myelomonocytic leukemia cell line WEHI-3 (Warner et al., 1979; ATCC No. TIB 68), murine embryonic fibroblast line NIH-3T3 (ATCC No. CRL 1658), human chronic myelogenous leukemia-derived line K562 (Lozzio and Lozzio, 1975, ATCC No. CRL 243), human ALL cell line Reh (Rosenfeld et al., 1975; ATCC No. CRL 8286), Jurkat cell line-derived line H33HJ-JA1 (ATCC No. CRL 8163), and monkey kidney cell lines Cos-1 (ATCC CRL 1650) and Cos-7 (ATCC CRL 1651) were obtained from ATCC. The murine cell lines NFS-60 and NFS-61 (Holmes et al., 1985), DA-3 (Ihle et al., 1984), and FDCP-1 (Dexter et al., 1980) were obtained from J. Ihle, Dept. of Biochemistry, St. Jude's Hospital, Memphis, Ten., U.S.A. Murine myeloid line 32DC123 (Greenberger et al., 1983) as well as murine T helper cell line TS1-C3 (Uyttenhove et al., 1988) and murine EL-4 thymoma line (Farrar et al., 1983) were a gift of L. Hültner, GSF Institut für Experimentelle Hämatologie, from whom also the L138.8A mast cell line was obtained (Hültner et al., 1989). The murine Friend erythroleukemia cell lines F4N (Ostertag et al., 1972; Dube et al., 1975) and B8/3 (Ostertag et al., 1973; Ostertag et al., 1974), were kindly given to us by W. Ostertag, Abteilung für Virologie, Heinrich Pette-Institut, Hamburg. The human T lymphoma cell line Jurkat was a gift of S. Thierfelder, GSF-Inst. f. Immunologie, Munich, and the murine bone marrow stromal cell line M2-10B4 (Lemoine et al., 1988) was obtained from C. Eaves, Terry Fox Institute, Vancouver, Canada. The

human bone marrow stromal cell line L88/5 was established by our institute from bone marrow of a normal donor by transfection with a replication-deficient SV-40 virus construct (Thalmeier et al., 1994). The CX-2 cell line is a moderately differentiated human colon carcinoma cell line established in 1979 as a xenograft in nude mice (Ovejera et al., 1979). It was a kind gift of G.Multhoff, GSF-Institut f. Klinische Hämatologie. The Burkitt's lymphoma cell line BL-70 was established by Lenoir et al. (1985) and given to us by G.W. Bornkamm, GSF-Inst. f. Klinische Molekularbiologie.

Mice :

All mice were obtained from the GSF in-house pathogen-free breeding facility. The following strains were used: C3H, Balb/c, CB1 (C57Bl/6), CBA, and AKR.

Cell culture media :

Media were purchased from Life Technologies, D-76339 Eggenstein. If not otherwise specified in the following, all cell lines were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, and 100 U/ml of penicillin and 100 µg/ml streptomycin (Life Technologies), respectively. This medium further contained 10% of fetal calf serum (FCS) obtained from different manufacturers and specifically tested with regard to particularly desired properties (e.g. promotion of differentiation-induction or optimal proliferation). To obtain WEHI-3-conditioned medium, a low serum medium was prepared from RPMI-1640 medium in analogy to the description by Guilbert and Iscove (1976). This medium contained 0.1% bovine serum albumine, 2 mM of L-glutamine, 20 U penicillin/ml, 20 µg/ml streptomycin, 32 mg/ml iron-saturated human transferrin, 10^{-5} M l- α -dipalmitoyl lecithin, 2×10^{-5} M oleic acid, and 2×10^{-5} M cholesterol. For the cultivation of Cos cells Dulbecco's medium containing 4.5 g/l D-glucose, 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin as well as 100 µg/ml of streptomycin was used.

For the cultivation of human bone marrow progenitors the BFU-E test was carried out in methyl cellulose cultures.

Methyl cellulose (Stem Cell Technologies Inc., Vancouver, Canada) was used in a concentration of 0.9% in Iscove's modified Dulbecco's medium (Life Technologies, D-76339 Eggenstein) containing 30% of selected fetal calf serum, 1% bovine albumin, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 1% of penicillin-streptomycin, 3 U/ml erythropoietin, 100 ng/ml rmu kit ligand, and either 10% of supernatant of human 5637 bladder carcinoma cell line (Takaue et al., 1987) or of a "cytokine cocktail". The "cytokine cocktail" was composed of 20 ng/ml rh GM-CSF, 20 ng/ml rh G-CSF, 20 ng/ml rh IL-3, and 20 ng/ml rh IL-6.

Bacterial strains :

Strains DH5 (Life Technologies, D-76339 Eggenstein) and Sure® (Stratagene, D-69044 Heidelberg) were used.

Bacterial media :

Bacteria were routinely cultured in LB medium (Sambrook et al., 1989). For transformation (Hanahan, 1983) also SOC medium was used as described by Sambrook et al. (1989).

Vectors :

As a prokaryotic-eukaryotic shuttle vector and as an expression plasmid for the preparation of a cDNA library, a 3,753 bp vector primer was used as principally described by Okayama and Berg (1982) which was obtained from USB company, Cleveland, Ohio, U.S.A. This plasmid, pXPRS+, (Pruitt, 1988) contains an SV-40 origin. Gene expression is controlled in eukaryotic cells by the SV-40 early promoter. In addition, a SV-40 polyadenylation site is contained. For stable transfections the 5,446 bp Rc/CMV vector (Invitrogen, Holland) was used in which transcription of the inserts is controlled via a CMV promoter and selection is carried out by a neomycin resistance gene.

Moreover, a cDNA library was established in lambda phages using vector Lambda Zap II® (Stratagene company, La Jolla, CA., U.S.A.). Using an appropriate bacterial strain, e.g. Sure®, this lambda phage enables blue-white selection,

and it contains a plasmid (pBluescript® SK-) which may be cut out of the λ phage together with the insert using helper phage R408.

Methods :

For removal of cells or organs from mice, the animals were sacrificed in ether narcosis. To obtain RNA, organs were immediately transferred to liquid nitrogen and after removal from the liquid nitrogen were further treated by crushing in the frozen state in a cooled mortar in the presence of added guanidinium rhodanide buffer. Bone marrow cells were flushed out from femoral and tibial diaphyses with a 12 gange needle using RPMI medium + 10% FCS and passed through a sieve. Spleen cells were obtained by crude cutting of the organs with scissors and passing through a sieve using a piston and addition of RPMI medium + 10% FCS.

Total cellular RNA was obtained by the acid phenol method according to Chomczynski and Sacchi (1987). From this material, mRNA was isolated if required by hybridization to oligo(dT) bound via streptavidin-biotin to magnetic particles (PolyAtract®, Promega-Serva company, D-69042 Heidelberg). Isolation of genomic DNA was performed using CsCl density gradient centrifugation with subsequent proteinase K and phenol treatment.

Highly competent bacteria ($\sim 1 \times 10^9$ colonies/ μ g DNA) were obtained following the protocol of Inoue et al. (1990) by cultivation of bacteria at 18°C up to an OD of 0.6. Bacteria were transferred to buffer containing $MnCl_2$ and $CaCl_2$ and stored with 7% DMSO in liquid nitrogen. Synthesis of cDNA by means of the pXPRS+ vector primer was carried out using the Clonstruct® kit of USB company, Cleveland, Ohio, U.S.A. according to manufacturer's instructions. To perform this, the poly(A) stretches of mRNA of WEHI-3 cells were bound to a poly(T) stretch in the opened vector-primer, and synthesis of the first and second strand was carried out on the vector-primer. Using terminal transferase, a poly(C) stretch was synthesized to the 5' end of the insert, and a complementary poly(G) strand was generated for ligation by

restriction of the 3' end with BstXI. Highly competent DH5 bacteria were obtained according to the description of Inoue et al. (1990) and transformed by these ligated vectors obtaining a total of 5×10^5 transformants. These were amplified in 1 x 500 ml of LB medium followed by isolation of plasmid DNA using CsCl density gradient centrifugation. An aliquot of the DNA bank obtained in this way was size-separated on an 1% agarose gel without previous restriction cutting. Using a supercoiled reference DNA (Life Technologies, D-76339 Eggenstein) plasmids showing insert sizes of 600 to 2,500 bp were cut from the gel and purified. The DNA obtained in this way was used for another transfection of highly competent DH5 bacteria in the course of the expression cloning.

To prepare the phage library from NIH-3T3 mRNA, the Time Saver® cDNA synthesis kit of Pharmacia Biotech company, D-79111 Freiburg was used according to manufacturer's instructions. To both ends of the cDNA NotI-EcoRI adaptors were ligated. The EcoRI restriction sites were ligated to those of EcoRI cut Lambda ZapII® phage of Stratagene company, La Jolla, CA., U.S.A. Subsequently, the phages were packaged using the Gigapack II® packaging kit of Stratagene company and were used to infect Sure® bacteria according to manufacturer's instructions. After selection of positive clones using a radiolabeled probe the inserts were excised from the lambda phage together with pBluescript® SK- using helper phage R408.

DNA gel electrophoresis was performed routinely on 0.8 to 1.5% agarose gels. DNA fragments were separated in low melting point agarose Sea Plaque GTG® (FMC Bio Products, Rockland, MD., U.S.A.) and then cut from the gel. Afterwards, the agarose was liquefied using GELase® (Biozym Diagnostik GmbH, D-31833 Hess. Oldendorf) according to manufacturer's instructions. Purification was carried out using Microcon 100® concentrators (Amicon GmbH, D-58453 Witten). Analytical fractionation of RNA was done using 2.2 M formalin-1.2% agarose gels. For hybridization, DNA and RNA gels were blotted onto Hybond-N® nylon filter membranes (Amersham-

Buchler, D-38110 Braunschweig) using a VacuBlot® device (Pharmacia Biotech, D-79111 Freiburg). Autoradiographic detection of distinct DNA or RNA bands on the nylon membranes was carried out by labeling the respective probes with ³²P dCTP using the Random Primed DNA Labeling kit of Boehringer Mannheim company. After washing of the hybridized filter membranes under the stringencies required according to the instructions of Sambrook et al., 1989, X-ray films were exposed to these filters. For quantification, Fuji imaging plates (Fuji Photo Film Co., Ltd. Japan) were exposed with the filters and digitalized by a Fuji phospho-imager and computer-evaluated. Sequencing of DNA fragments was done by the chain termination method according to Sanger et al. (1977) using di-deoxynucleotides.

Transfection of Cos-1 and Cos-7 cells was done by the DEAE dextran method (Cullen, 1987). After 30 min of incubation of the cells with 500 ng of DNA/3.5 cm² culture disk and 5% DEAE dextran, and after incubation for 2.5 hr with 80 µM chloroquine an incubation was performed for 3 minutes with 15% buffered glycerol instead of performing the DMSO shock mentioned in the protocol. By this, a substantially decreased background of differentiation induction was achieved by the Cos cell supernatants. The conditioned Cos supernatants were harvested after 72 hours, and were added in serial dilutions with initially 50% of murine erythroleukemia cell supernatants of lines F4N or B8/3 in 96-well microplates. Induction of differentiation in these samples was determined by counting the percentage of benzidine-positive cells after 3 and/or 4 days. For this purpose, a stock solution of 10 mg of N,N,N',N'-tetramethylbenzidine (Sigma Biochemicals, D-82039 Deisenhofen) in 10 ml 12% acetic acid (NTMB solution) was prepared. Immediately before staining was carried out, a dilution of 35 µl NTMB solution and 35 µl of isopropanol and 5 µl of 30% H₂O₂ was prepared. Depending on the cell density, 5 - 10 µl of the cells were pipetted into a new microwell and filled up to 100 µl with fresh RPMI 1640 medium containing 10% FCS. 5 ml of the diluted NTMB solution were added.

Following an interval between 10 and 30 minutes, the percentage of benzidine-positive cells was counted in the microwells by their green color using a reverse microscope.

Stable transfection of CX-2 cells was done using the LipofectAMINE® transfection kit of Life Technologies company, D-76339 Eggenstein, according to manufacturer's instructions. 4 µl of LipofectAMINE® were used per well of a 6 well plate (= 10 cm², Nunc GmbH company, 65203 Wiesbaden).

To prepare WEHI-3-conditioned media the cells were inoculated three days prior to the preparation in a density of 2.5×10^4 /ml in RPMI 1640 medium containing 10% FCS and the other usual additions. After 3 days the cells had grown to densities between 3.5×10^5 and 1.2×10^6 /ml. They were centrifuged and washed 1 x in RPMI 1640 medium without any additions and then adjusted to 1×10^6 /ml in low serum medium. After 3 days, the conditioned medium was harvested and following a vigorous centrifugation was 10fold concentrated using an Amicon® 10 concentrator. These WEHI-3-conditioned media (WCMs) were aliquoted and frozen at -20°C. In this form, the supernatants could be stored over 3 years with a 2 to 8fold loss of activity occurring gradually.

For fractionation, the WCM concentrated by 20 to 50fold was separated into single fractions by gel filtration on Sephacryl S300® using a gel bed of 90 x 2.6 cm with PBS as running buffer and a flow rate of 10 ml/min with detection being performed at 280 nm. BSA (68 kDa), chymotrypsinogen (25 kDa), and cytochrome c (12.5 kDa) served as molecular weight standards.

The relative cell number per microwell in 96 well microplates was determined using the MTT test (Mosmann, 1983). This test detects the ability of cells to convert the yellow-colored tetrazolium salt of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) into the purple MTT formazan. This ability is dependent on dehydrogenases in active mitochondria whereby eventually the cell number is evaluated relative to the number of active mitochondria. Color intensity was determined with an ELISA reader (SLT, Salzburg) using a test wave length of 550 nm and a reference

wave length of 690 nm.

Adherence of WEHI-3 cells was tested after incubation with WCM by their adherence to the plastic bottoms of 96 well microplates (Nunc GmbH, 65203 Wiesbaden). To perform this, the supernatant was removed from the respective microwells and the bottom of the microwells was rinsed 1 x with fresh medium (RPMI 1640 without additions). After aspiration of the medium containing the detached cells in the supernatant fresh RPMI 1640 medium containing 10% FCS and 2 mM L-glutamine was added and the MTT test performed. Cells which were detached and already floating in the supernatant were pipetted into new microwells and measured by the MTT test in a similar fashion.

For the isolation of CD34+ cells from human bone marrow Percoll-separated mononuclear bone marrow cells were incubated with paramagnetic plastic beads (Dynabeads®) coated by monoclonal antibody BI-3C5 directed against CD34 (Deutsche Dynal GmbH, Hamburg). The CD34+ cells attached to Dynabeads® were separated by a permanent magnet, and afterwards the Dynabeads® were removed from the CD34+ cells by treatment with a specific antibody preparation obtained from the same company (DETACHaBEADS®). The whole procedure was carried out according to the instructions of Dynal company.

Colony numbers in the cultures of human CD34+ bone marrow cells were evaluated after 14 - 21 days using a inverted microscope. In the evaluation, all hemoglobinized colonies and those with similar morphology but lacking the hemoglobin staining were counted as BFU-E, all colonies which did not contain BFU-E were summarized as GM-CFC, and those colonies which were formed from at least 2 different cell types were counted separately as mixed colonies.

Results

EDA activity in different cell lines :

Fig. 1 shows the effects of 4 different WEHI-3-conditioned media on the number of benzidine-positive F4N cells and, thus, on the differentiation and hemoglobinization of these cells. The number of benzidine-positive cells upon

incubation with 1.2% DMSO is shown as a positive control. With DMSO, up to 70% of F4N cells become positive on day 4. However, despite a similar amount of differentiation induction by WCM in this experiment, its kinetics differ from that of DMSO: Differentiation is induced in these cells by WCM not before day 3 which is clearly different from the negative controls. This activity in WCM as measured on murine Friend erythroleukemia cells was designated by "EDA" for Erythroid Differentiation Activity as a working designation. The amount of differentiation induction using the same WCM was subject to greater variations in individual experiments which may be attributed to the differentiation tendency of the erythroleukemia cells. Factors of the medium, the FCS, as well as the initial cell density of the erythroleukemia cells played a role. Therefore, a reference WCM was included in such examinations as a positive control. EDA was not only detected in the WEHI-3 cell supernatant but also in the supernatants of NIH-3T3 cells upon cultivation in 1% FCS, and also in supernatants of the human bone marrow stromal cell line L88/5, although in the latter case only after irradiation as well as a prolonged culture period (Fig. 2).

Effect of EDA on murine and human cells :

The factor having an effect on murine cells may be of murine or human origin. The murine factor in WCM also shows a weak effect on human K562 cells which are not induced to erythropoietic differentiation by DMSO (Fig. 3, especially WCM (C)). Therefore, the activity referred to as EDA is species-crossing in both directions.

Biological properties of the activity studied :

The effect of EDA on murine erythroleukemia cells is not primarily directed to the inhibition of proliferation. Although in many experiments a proliferation inhibiting effect upon higher factor concentrations was of considerable importance, experiments as e.g. presented in Fig. 4 show that while an induction of differentiation clearly occurred as may be seen from the increase in α -globin mRNA expression no

effect on the proliferation could be observed. This differentiation induction was accompanied by a down-modulation in the amount of c-myb transcript which was also observed if incubations of other cell types such as 32DC123 with WCM were performed, and which, moreover, was accompanied by a concentration-dependent prolongation of the half life of c-myb mRNA. Since a down-modulation of c-myb may be observed in many hematopoietic cellular systems in the course of differentiation, it may be assumed that the induction of erythropoietic differentiation by EDA only represents the effect on one of several cellular systems. In this respect, the increase in WEHI-3 cell adherence by their own conditioned medium (Fig. 5) which has been detected by us may possibly be considered as an auto-inductive effect in the sense of an induction of differentiation. In this context, the increase in adherence of these myelomonocytic cells is equivalent to a differentiation step towards the development of macrophages. According to these findings and assuming that the effects have also been brought about by EDA there is the possibility that EDA may be a factor which generally induces differentiation at least in hematopoietic cells.

Physical and chemical properties of EDA :

Some of the physical and chemical properties are summarized in Table 1.

Exclusion of individual cytokines as effectors of the EDA effect :

Theoretically, a number of cytokines may be the effectors of the activities observed in the differentiation assay with murine erythroleukemia cells. Several cytokines could be ruled out as effectors. (Table 2).

Fractionation of WEHI-3-conditioned media :

WEHI-3-conditioned media were fractionated by means of Sephacryl S300® gel filtration. After addition of 0.05% BSA, the individual fractions were incubated in a volume of 20% with B8/3 cells in 5 ml cultures for 4 days. Afterwards, the relative amount of α -globin mRNA formed was quantified. The main peak in Fig. 6 ran in the low molecular weight slope of BSA.

Screening for optimal EDA expression-conditions to be used in expression cloning :

WEHI-3 cells were grown to different densities in 250 ml flasks with FCS-containing medium. After reaching the various densities (Fig. 7), a major portion was harvested for mRNA preparation and a minor portion was again cultivated under standard conditions in low serum medium for 3 days to obtain WCM. As shown in Fig. 7 the desired densities of approx. 2.5×10^5 (W3/1), 5×10^5 (W3/2), and 8×10^5 cells/ml (W3/3) were reached after different times. The cells were adjusted to a uniform density of 1×10^6 /ml and were further cultivated for 72 h in low serum medium. The highest EDA activity was found in the W3/2 supernatants (Fig. 7). Therefore, the RNA obtained from the harvested W3/2 cells was further treated to construct an expression library. It becomes clear from Fig. 7 that always an inhibition of the differentiation induction is found if higher WCM concentrations are used. At the same time, an inhibition of the proliferation is observed for the same concentration. Fig. 8 shows that for the W3/2 WCM the optimal concentration for an induction of differentiation in B 8/3 cells by fractions between 66 and 25 kDa is at 25%. Although in this analysis in accordance to the results of Fig. 6 the main EDA peak was found at a molecular weight between 60 and 40 kDa other similar studies showed significant activities down to molecular weights of 10 kDa. Possible explanations are the presence of different molecules which show EDA activity or the association of one EDA molecule species in aggregates of different sizes or the formation of degradation products.

It was observed during the analysis of optimal culture conditions for WEHI-3 cells to obtain high EDA activities that the type of primary incubation in 10% FCS is of importance. Tests of 6 different batches of FCS obtained from various manufacturers gave very different yields of EDA activity. The most promotive FCS was used for further experiments. Another important finding was that it was impossible to obtain EDA activity if in the second cultivation step (typically 72 h in low serum medium containing 0.1% BSA) the same medium containing 10% FCS was used as in the first step (data not shown).

Construction of an expression library using W3/2 RNA and vector pXPRS+ :

The library prior to amplification comprised 5×10^5 clones. After amplification and size selection (inserts of >600 bp) approx. 500 clones per each circular Hybond-N® filter with a diameter of 14 cm were plated onto LB agar. For isolation of plasmid DNA, clones were grown by transfer on a second filter. After transfection of this DNA into Cos-1 cells, the Cos-1 supernatant was tested in a sample with B 8/3 cells. The selection criterion was the percentage of benzidine-positive cells after 3 - 5 days. A total of 25,000 clones were screened in this way. The D series showed a positive signal (Fig. 9). The 520 series D clones were divided into groups each with approx. 25 clones and were retested (D15 series). Then, a step with approx. 10 clones in each group (DX series) was carried out followed by testing each of the remaining 10 clones individually (DY series). The activity obtained with clone DY-8 was 1-2 dilution steps lower than that of the positive WCM control. The DNA of this clone was purified and transfected transiently into Cos-1 cells in 3 independent experiments. A highly significant difference was obtained between the differentiation induction by supernatants of cells transfected with DY-8 and the differentiation induction achieved by control supernatants after transfection with the same vector containing an irrelevant insert of the same size (Fig. 10).

Radioactive screening of the pXPRS+ library with clone DY-8 :

Instead of a distinct band, a smear over approx. 200 bp appeared for clone DY-8 which, thus, included a size range of about 950-750 bp. Because it was supposed that a truncated clone had been obtained, the gene library was screened using radiolabeled DY-8 as a probe. A total of 32 clones was obtained the largest being about 1,350 bp in size. This clone was referred to as HA-15/2. It exhibited a weak differentiation-inducing effect for which, however, no statistical significance was achieved in 3 experiments performed (Fig. 11). Because of its size it served as a radiolabeled probe in the further detection operations performed by hybridization (Southern blotting, Northern blotting, additional screening of the gene library) although it was assumed that it was an incomplete clone.

Establishing a cDNA library in lambda phages :

Assuming that the strongest eda activity was derived from a 2.2 kbp mRNA species which is particularly highly expressed in NIH-3T3 cells (see below) cDNA was transcribed from NIH-3T3 poly(A)+ mRNA and size-separated on a non-denaturing gel. The 1,900 to 2,500 bp region was cut from the gel, purified, and cloned into Lambda Zap II® phage. However, all eda clones obtained by this procedure were smaller than 1,500 bp.

Southern blot analysis :

Analyses of genomic DNA from the spleen of C3H mice in hybridization experiments using the 1,350 bp probe HA-15/2 (BamHI fragment) showed 4 bands of 6.5, 5.7, 3.8, and 2.1 kbp particularly after digestion of the DNA with SacI. Using EcoRI, 2 weak bands at 7.5 and 5.5 kbp were obtained in the DNA of WEHI-3 cells. The observation that also human genomic DNA obtained from cell line K562 after digestion with EcoRI showed a band at 7.5 kbp and another band at 6.5 kbp (Fig. 12) was important in this respect. Since highly stringent washing conditions were used (up to 2 x 30 min 0.1 x SSC/0.1% SDS at 60°C) some homology should be present between the

murine and the human gene. Fig. 12 proves that it is possible to obtain the human eda gene from a gene library using the radiolabeled murine probe.

Studies of the eda gene expression :

The experiments were carried out using the approx. 1,350 bp BamHI fragment of HA-15/2 as a probe. The Northern blot analysis after stringent washing (up to 20 min 0.1 x SSC/0.1% SDS and 60°C) reveals several bands of varying size showing different hybridization intensities (Fig. 13). Single bands of more than 5 kbp vary between the cell types (see below). Generally, the band with the highest intensity appeared at 2,200 bp with further bands in varying intensities appearing at 1,750 bp, 1,350 bp, and 1,200 bp. Fig. 13 shows that all of the bands hybridize equally well to the complete probe (BamHI fragment of HA-15/2) and to the 500 bp of the 3' end of this probe, but that in contrast the 200 bp from the 5' end of this probe gives a detectable signal only with the larger bands of more than 5,000 bp and with the 2,200 bp band. From this it may be concluded that the large bands contain at least portions of all of the smaller bands and the 2,200 bp band as well as bands > 2,200 bp contain a detectable portion of the 5' end of the probe while the 3' end of the probe is common to all bands. An examination of several mouse strains regarding the band variations in the 2 cell types NIH-3T3 and M2-10B4 (Fig. 12) revealed that within a strain all bands are identical in different tissues but that the bands vary between the mouse strains. NIH-3T3 cells are fibroblasts derived from Swiss mice while the bone marrow fibroblasts of line M2-10B4 are derived from B6C3F₁ mice (Lemoine et al., 1988). Analogously, on the genomic DNA level there are a number of differences in restriction between individual mouse strains (data not shown).

Expression pattern of eda in normal mouse tissues :

The expression pattern of different tissues was studied with C3H mice. The highest expression was found in normal thymus (approx. similar for adult and fetal (d 15)), and in

fetal liver (d 15). Next with regard to the amount of expression was the spleen while the expression in normal bone marrow was significantly weaker. A very weak expression which in some occasions was only detectable at the poly(A)+ level was present in all organs and tissues examined such as liver, kidney, intestine, brain, and placenta. In contrast, primary mast cells isolated from bone marrow by 4 weeks of culture of the bone marrow in the presence of IL-3 were more positive than normal spleen cells.

Expression pattern in murine cell lines :

The amount of expression in the cell lines varied greatly but generally was stronger than in the primary tissues. Among the non-malignant cells the embryonic fibroblast line NIH-3T3 revealed a strong expression while the L929 fibroblast line almost showed no expression at all. A median expression was observed for the M2-10B4 bone marrow fibroblast line. Very strong expression was found for the T helper cell line TS1-C3 while for the EL-4 thymoma cell line almost no expression was observed. Myeloid FDCP-1 and 32DC123 cell lines as well as the 138-8A mast cell line showed only weak expression. Generally, the cell lines established from murine malignant hematopoietic tissues had a strong to very strong expression. This was obvious for WEHI-3 cells, DA-3, NFS-60, and NFS-61 (all cell lines derived from murine leukemias).

Expression pattern in human cells :

Using HA-15/2 as a radiolabeled probe, it is possible to detect eda expression in human cells although with considerable background. The background relates to a strong co-hybridization of ribosomal 28S RNA so that stringent washing for up to 20 min in 0.2 x SSC/0.1% SDS at 60°C has to be carried out to detect the bands of interest. In particular, a transcript of 2.5 kbp was found in the poly(A)+ RNA of Jurkat cells, and also in K562 total cellular RNA a weak band of that type can be observed (Fig, 14). Regarding the lower molecular weight eda mRNA species, up to now only

in one case of a T-CLL a distinct band of 1,100 - 1,200 bp was found with the murine probe (data not shown).

Studies of eda mRNA expression in murine spleen cells :

During in vitro incubation (1×10^6 cells/ml in RPMI 1640 containing 10% FCS and the usual additions) spleen cells lose their eda expression within a few hours. Upon addition of anti-T cell receptor antibody or concanavalin A (Fig. 15), or also of TPA a slight increase of the 2,2 kbp band as well as the larger bands is achieved within one hour while after only 4 more hours those transcripts have again decreased with accumulation of eda degradation products of defined sizes (< 400 bp) predominating without stabilization of the normal transcripts. These effects largely wear off within a period of 24-28 hours.

However, a stabilization of eda mRNA in spleen cells in vitro was achieved upon performing a mixed spleen cell reaction for 3 days using in each case 1×10^6 non-irradiated and untreated CBA and CBL cells/ml (Fig. 16). Again, no mRNA stabilization was achieved if the CBA spleen cells were previously irradiated with 15 Gy and also if CBL spleen cells alone were cultivated in vitro.

Involvement of eda in the allogenic reaction which interestingly took place only if stimulation was carried out with non-irradiated cells (Fig. 16) was confirmed in an in vivo model of acute graft-versus-host (gvh) disease. If 5×10^7 CBL spleen cells are injected into (CBA x CBL) F_1 hybrids after carrying out a 9 Gy whole body irradiation a severe gvh disease develops. We found that in the course of this disease on day 6 after transplantation in the spleens of the recipient animals an increase in eda expression by approx. 7fold occurred as compared to the controls in which the eda expression decreased (Fig. 17). This reaction was restricted to spleen presumably because in the other organs the percentage of inflammatory cells participating in the gvh disease relative to all cells was too low to bring about a detectable increase in eda expression in the Northern blot analysis.

eda sequence analyses :

Analysis of the DNA sequence of eda or the different cDNA species, respectively, turned out to be difficult in particular because of the presence of many repeat structures as well as AT- and GC-rich stretches. A consensus sequence was derived from different clones of the pXPRS+® and Lambda Zap II® libraries (Fig. 18) which most likely is a part of the 2.2 kbp cDNA. Fig. 19 shows the presumable structure of the 2.2 kbp sequence which has not yet been completely analysed. Three different repeat types are indicated by R1, R2, and R3. The regions indicated by bold lines have been confirmed with respect to their relative assignment. However, it is not clear whether the approx. 300 bp which are still missing reside in the beginning part of the sequence as indicated in Fig. 18 or whether another repeat of already known sequence is present. No open reading frame was found, the frequent stop codons (as indicated by *) appearing preferably in the codons at the beginning of the repeats. Not all of them are represented in Fig. 19. The larger repeats occurring in this sequence are summarized in Table 3.

Fig. 20 shows the sequence of clone DY-8 in each of the upper of the 2 rows. This sequence contains an open reading frame starting with the boxed ATG at position 155, however the activity of this clone after transfection into Cos cells is not strong enough to suggest that this may be the critical 5' end of the open reading frame. The consensus sequence with the presumptive 2.2 kbp eda sequence of Fig. 18 starts at position 74. Thus, only the first 73 bases at the beginning represent DY-8 specific sequence. Since the 5' end is not unambiguously defined a longer reading frame has been created by inserting an ATG at position 52 (Fig. 20, lower row) including the entire sequence in common with the 2.2 kbp eda sequence. In contrast to the shorter open reading frame of DY-8 (534 bp eda) this has been indicated as 636 bp eda.

Transfection of eda sequences using vector Rc/CMV :

Each of the 534 bp and the 636 bp eda fragment (see previous chapter) were ligated into vector Rc/CMV and stably

transfected into human colon carcinoma cell line CX-2. Supernatants of this cell line were obtained 6 weeks after transfection under permanent G 418 selection upon cessation of the antibiotic and tested for their ability to induce the differentiation of murine erythroleukemia cells of line B8/3. Fig. 21 demonstrates that similar to the results shown in Fig. 10 the 534 bp eda containing the open reading frame of clone DY-8 exhibits a differentiation-inducing activity on this cell line of a strength similar to that of the WEHI-3-conditioned control medium while the longer fragment transfected (636 bp eda) has an activity which is 1.5 - 2 dilution steps higher.

Growth factor properties of eda on cell line BL-70 :

The human BL-70 cell line is derived from an EBV-negative Burkitt's lymphoma and has been established in culture by Lenoir et al. in 1985. In 1993, Falk et al. performed a detailed study of this cell line and showed that up to a certain density it is not able to grow in the absence of stromal cells. However, exceeding a critical cell density the cells are able to proliferate free of stromal support. As shown in Fig. 22 small volumes of the supernatant of a clone of cell line CX-2 transfected by the 534 bp eda are able to convert the density-dependent growth of the control into a density-independent growth.

Effect of eda on CD34+ progenitors of human bone marrow :

In several experiments the effect of eda-containing supernatant on progenitors of human bone marrow was tested (Fig. 23). Effects on early progenitors (mixed colonies in Fig. 23) as well as more mature progenitors (BFU-E and GM-CFC in Fig. 24B) are noticeable. They were directed to the bone marrow of a hematologically normal individual as well as bone marrow of a patient suffering from chronic myelogenous leukemia (Fig. 23B). These effects may be interpreted both as effects of a growth factor and of a survival factor.

It has been discovered according to the invention that the quality of the protein provided, i.e. its activities

depend particularly on the 3' end of the nucleotide sequence encoding the protein while the quantity of protein expression is affected by the 5' end.

As demonstrated above it has not been possible to determine the exact 5' end. However, on the basis of the results described herein it is easily possible for the skilled artisan to determine or to select, respectively, the 5' end(s) to enable a higher amount of expression.

The invention comprises all proteins having the properties described regardless of their appearance in animal or in human cells. The DNA and amino acid sequences presented are in part consensus sequences, i.e. several sequences have been used to derive the sequence which leads to a continuous reading frame. It can not be excluded that the native protein differs from the polypeptide encoded by the consensus sequence.

Claim 1 of the present application characterizes the protein by 8 different features a) to h). The protein provided by the invention has to comply with at least the features a), c), and d). At least one or more of the additional features are fulfilled in preferred embodiments of the invention.

Table 1:

Percentage of reduction of EDA activity

1.	Trypsin digestion: 50 μ g/ml 30 min at 37°C:	75.7%
2.	Cleavage of disulfide bonds: 50 mM DTT 2 h at room temperature	98.5%
3.	Heat inactivation: 60°C, 20 min:	0%
4.	Heat inactivation: 80°C, 20 min	61.7%
5.	Freeze/thaw cycles (5 cycles):	37.1%

Table 2:

Cytokins without differentiation-inducing activity on murine
erythroleukemia cells

Cytokine	Cells tested	Method	Result
Epo	F4N	Benzidine	neg.
IL-3	F4N	Benzidine	neg.
IL-6	F4N, B8/3	α -globin mRNA	neg.
LIF	F4N, B8/3	α -globin mRNA	neg.
TNF- α	F4N, B8/3	α -globin mRNA	neg.
TGF- β	F4N, B8/3	α -globin mRNA	neg.
Kit Ligand	B8/3	Benzidine	neg.

Table 3: Repeat structures of the presumptive consensus partial sequence of the 2,200 bp eda cDNA

1. 42 bp Repeat

855 CGTCCGCCGG TCACGGCCGC CGCCCCCAGC GACGTCACCC AC

Repetition of 1248 - 1289

2. 55 bp Repeat

903 AGAAGCGGAC GCCGCGGTCA AGATGTCTCT GCCATGCCCA CGGGACGCAC
953 GGACG

Repetition of 1304 - 1358

3. 81 bp Repeat

163 TAGTCCTGCC GTCGTCAATG GTTCTCTATG GGCTTTCAGA GTGAGTGGCG
213 GGAAGGCGGC CCCGAGGCAT GCTGGGAGTT G

Repetitions of 82 - 162; and of 244 - 324

4. 178 - 180 bp Repeat

348 GTTCTCTGT ATAGACCTGG CTGTGGATTT TTCGCTAATT CTTTTTTTTA
398 GCTTTATTTT TAATTTTAC TTTTTCACAC AGGATTCTC TTTATAGCCT
448 TGGCTACCGT TTTTCCCTA ATTATTCTCC TTTTCATTTT GGTTTATTTT
498 TTTTAAATTT TGGTTTTTTT AAGACAGG

Repetition of 526 - 705

5. 385 bp Repeat

1001 GACCGCACCG CCGCACCCAC CACAGCACAC AGGACACACG AGGA AGCCTGCGCC
1051 GCCCGCCCAG GCACACGCGG CACACACGGC ACACACGGCA GGCAGGCCAG
1101 GCACACGCAT CCGCAGGACC CGCCGCACCC GCCACGCAGA CACGGACGAG
1151 CCGCCGCGGT CAAGATGTTC ACCCGCCGCG GTCAAGATGT ATGTGCCACC
1201 GACCCTCGCC CCGCTGGACG GACGGACGGA CGCACGCACG CCGTCAGCGT
1251 CCACCGGTCA CTGCCGCCGC CCACAGTGAT GTCACCCACG AAAGCACACA
1301 CGTAGAAGCG GACGCCGTGG TCAAGATGTC TCTGCCATCC CCACAGGACG
1351 GACGGACGGA CTCCACAAGG T

Repeated only in single clones, does not appear in the consensus partial sequence

Novel protein having at least differentiation-inducing
activity on Friend erythroleukemia cell lines

C L A I M S

1. Protein with at least differentiation-inducing activity on Friend erythroleukemia cell lines having the following properties wherein said protein has to comply with at least the features a), b), c), and d):
 - a) may be isolated from murine myelomonocytic leukemia cell lines;
 - b) may be isolated from irradiated human bone marrow stromal cell lines;
 - c) induces differentiation in Friend erythroleukemia cell lines with hemoglobin formation;
 - d) having a molecular weight in the range of about 10 - 60 kDa as determined by gel filtration on Sephacryl S300®;
 - e) with an expression of the corresponding mRNA in primary cells of the thymus, fetal liver, adult spleen, or bone marrow;
 - f) with characteristic repeat structures in the cDNA encoding the protein;
 - g) with corresponding mRNA species of different length consisting of identical 3' regions but different 5' regions.
2. Protein according to claim 1, characterized in that said protein has at least one of the following features:
 - h) showing a stable in vitro expression of the corresponding mRNA if an allogenic spleen cell reaction is carried out with non-irradiated, not pretreated spleen cells of mouse strains CBA and C57Bl/6;
 - i) having AT rich regions in the cDNA encoding the protein;
 - k) inducible by a serum factor present in fetal calf serum.
3. Protein according to claim 1, characterized in that

one or more of the repeat sequences presented in Table 3 or of repeat sequences hybridizing to these repeat sequences under stringent conditions are present in the DNA encoding the protein of claim 1 or claim 2.

4. Protein according to one or more of the preceding claims,
characterized in that
said protein may be isolated from human cells, murine cells, or the culture supernatants of human or murine cell lines.
5. Protein according to one or more of the preceding claims,
characterized in that
said protein exhibits a partial amino acid sequence encoded by a DNA hybridizing to the cDNA of SEQ ID NO:1 or NO:2 or NO:4.
6. Protein according to one or more of the preceding claims,
characterized in that
said protein exhibits a partial amino acid sequence encoded by a DNA hybridizing to the cDNA of SEQ ID NO:1 or NO:2 or NO:4 under stringent conditions.
7. Protein according to one or more of the preceding claims,
characterized in that
there are also comprised portions, analogues, and derivatives of said protein as well as fusion proteins each coding for a protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines.
8. Protein according to one or more of the preceding claims having an essentially purified, native form.

9. Protein according to one or more of the preceding claims having an essentially recombinant form.
10. Protein according to one or more of the preceding claims said protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines and/or growth factor activity and/or colony-stimulating activity.
11. Protein according to one or more of the preceding claims,
characterized in that
said protein exhibits a differentiation-inducing effect on human leukemia cell lines.
12. Protein according to one or more of the preceding claims,
characterized in that
said protein contains partial amino acid sequences according to SEQ ID NO:3 or NO:5 wherein one or more of the amino acids may be deleted, substituted, or added each having at least differentiation-inducing activity on Friend erythroleukemia cell lines.
13. DNA fragment according to SEQ ID NO:1 or NO:2 or NO:4 or the complementary strand thereof, portions, derivatives, and analogues thereof each coding for a polypeptide having at least differentiation-inducing activity on Friend erythroleukemia cell lines.
14. DNA fragments, portions, analogues, and derivatives thereof each coding for a polypeptide having at least differentiation-inducing activity on Friend erythroleukemia cell lines hybridizing to the cDNA according to SEQ ID NO:1 or NO:2 or NO:4 and/or which are degenerated by the genetic code.
15. DNA fragments, portions, analogues, and derivatives

thereof each coding for a polypeptide having at least differentiation-inducing activity on Friend erythroleukemia cell lines hybridizing to the cDNA according to SEQ ID NO:1 or NO:2 or NO:4 under stringent conditions and/or which are degenerated by the genetic code.

16. DNA fragment of one or more of the preceding claims, characterized in that said DNA fragment encodes at least a part of a polypeptide with the activity of the human or murine protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines according to one or more of the preceding claims.
17. Recombinant vector, characterized in that said vector contains a DNA sequence corresponding to a gene or a DNA fragment encoding the protein with at least differentiation-inducing activity on Friend erythroleukemia cell lines according to one or more of the preceding claims.
18. Recombinant vector according to one or more of the preceding claims, characterized in that said vector is derived from a bacterial plasmid, a bacteriophage, or a viral vector.
19. Host cell transformed by a vector according to one or more of the preceding claims.
20. Host cell according to claim 19, characterized in that said host cell is a prokaryotic cell or an eukaryotic cell.
21. Method for the preparation of a DNA fragment according

to one or more of the preceding claims,
characterized in that
said fragment comprises screening of a human or murine
cDNA clone library using as a probe a DNA fragment of a
DNA coding for a murine or human protein having at least
differentiation-inducing activity on Friend
erythroleukemia cell lines.

22. Monoclonal or polyclonal antibody directed against at least one epitope of a protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines according to one or more of the preceding claims.
23. Therapeutic, diagnostic or experimentally useful means, characterized in that
said means contains as an effective substance at least one nucleic acid in an effective amount which hybridizes to a gene or a part thereof encoding the protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines according to one or more of the preceding claims.
24. Means according to one or more of the preceding claims, characterized in that
said means contains as an effective substance at least one nucleic acid comprising (a) the nucleotide sequence encoding a protein with at least differentiation-inducing activity on Friend erythroleukemia cell lines, (b) a portion thereof, (c) a nucleotide sequence hybridizing to a nucleic acid as under (a) and/or (b) under stringent conditions, or (d) a nucleotide sequence complementary to a nucleotide sequence as under (a), (b), and/or (c).
25. Means according to one or more of the preceding claims, characterized in that
said nucleic acid optionally is a modified DNA.

26. Means according to one or more of the preceding claims, characterized in that said nucleic acid optionally is a modified RNA.
27. Therapeutic means, characterized in that said means contains a protein, an analogue, a derivative or portions thereof according to one or more of the preceding claims each functioning as a polypeptide with at least differentiation-inducing activity on Friend erythroleukemia cell lines together with conventional carriers and excipients in an effective amount.
28. Use of a means according to one or more of the preceding claims as a molecular probe in diagnostics or therapy.
29. Use of a means according one or more of the preceding claims as an antisense nucleic acid for the inhibition of gene expression.
30. Use of a DNA encoding a protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines, a portion, derivative, or analogue thereof each functioning as a polypeptide with at least differentiation-inducing activity on Friend erythroleukemia cell lines for the incorporation into a prokaryotic or eukaryotic cell.
31. Fusion protein having an amino acid sequence consisting completely or in part of the amino acid sequence of the human or murine protein with at least differentiation-inducing activity on Friend erythroleukemia cell lines according to one or more of the preceding claims and in part of a prokaryotic and/or eukaryotic protein.
32. Synthetic protein having at least differentiation-inducing activity on Friend erythroleukemia cell lines

33. Use of a protein according to one or more of the preceding claims or of inhibitors of said protein in the treatment of diseases in which a local or systemic overproduction or underproduction of this protein affects the development of the disease or the course thereof.
34. Use of a protein according to one or more of the preceding claims as a growth factor, colony-stimulating factor, a factor inducing erythropoiesis and/or inducing the immune system.
35. Protein according to one or more of the preceding claims,
characterized in that
said protein comprises at least those amino acids which
are encoded by nucleotides 74 - 154 or 155 - 685.

S U M M A R Y

According to the invention, there is disclosed a novel protein having at least differentiation-inducing activity in particular on Friend erythroleukemia cell lines.

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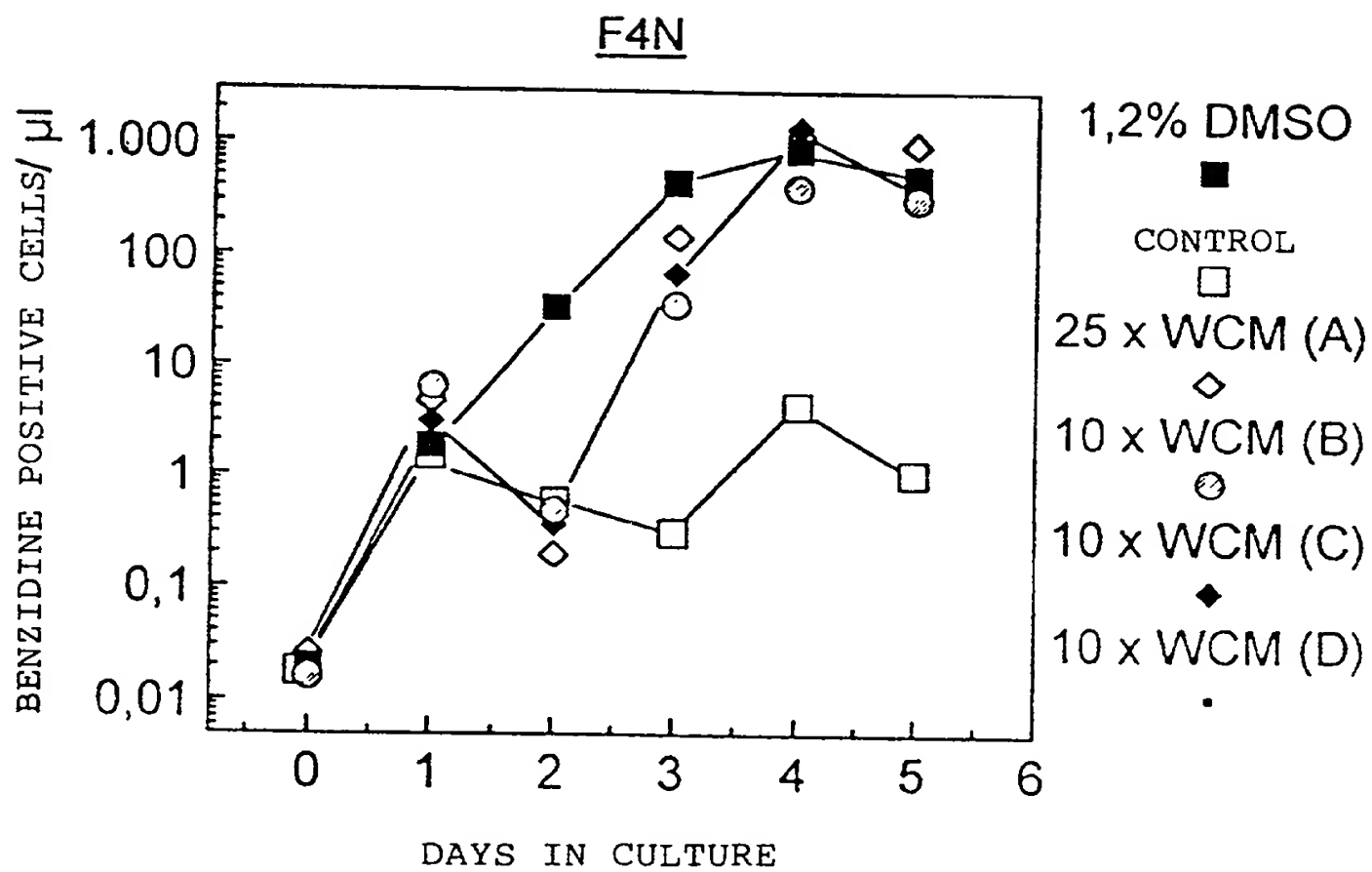


FIGURE 1

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
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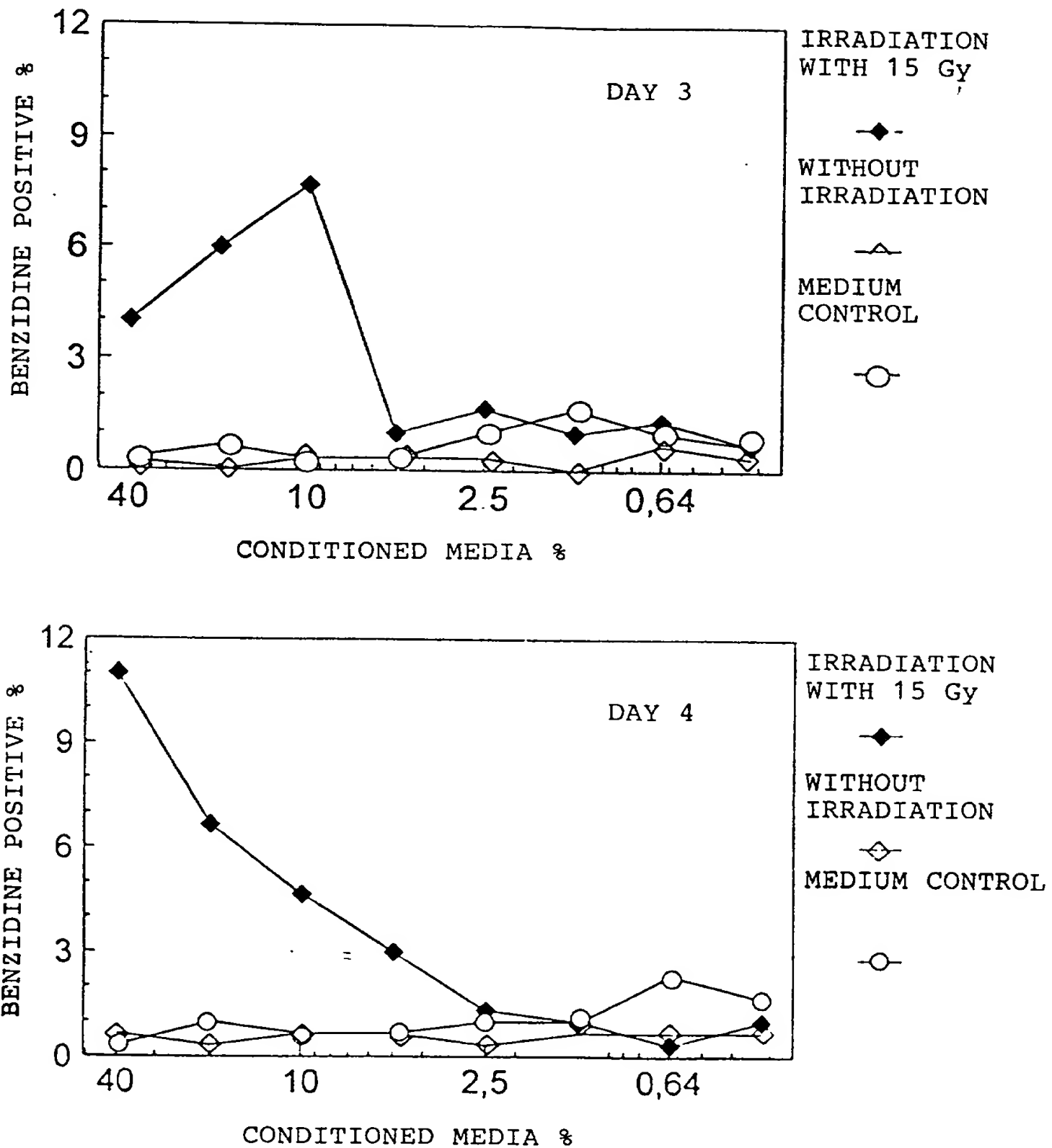


FIGURE 2

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
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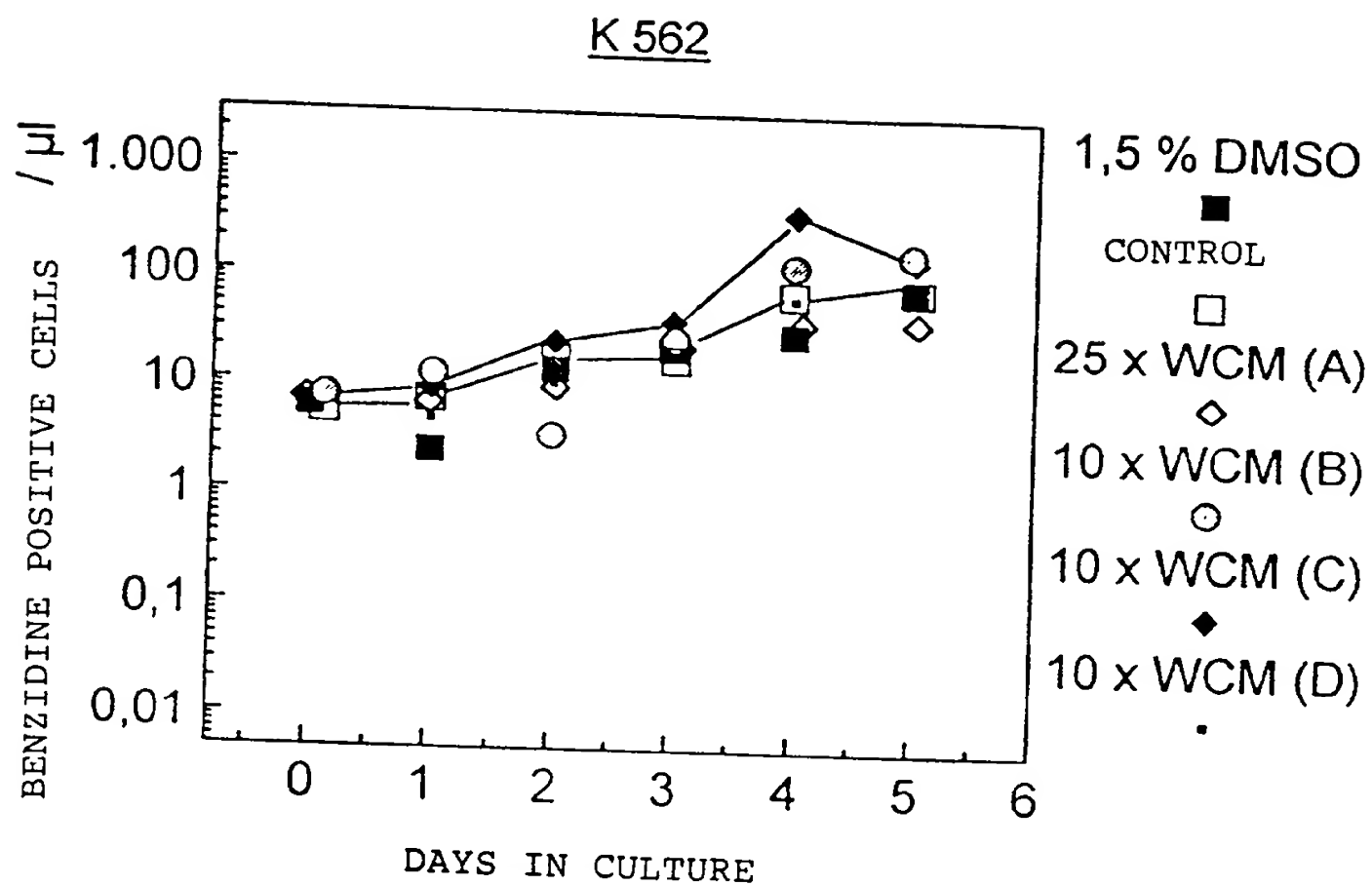


FIGURE 3

APPROVED	O.G. FIG.	
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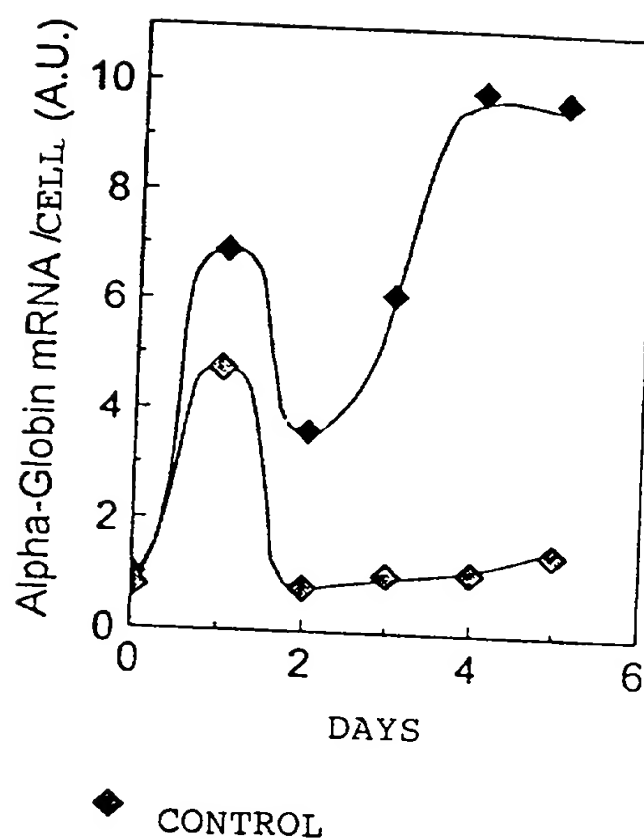
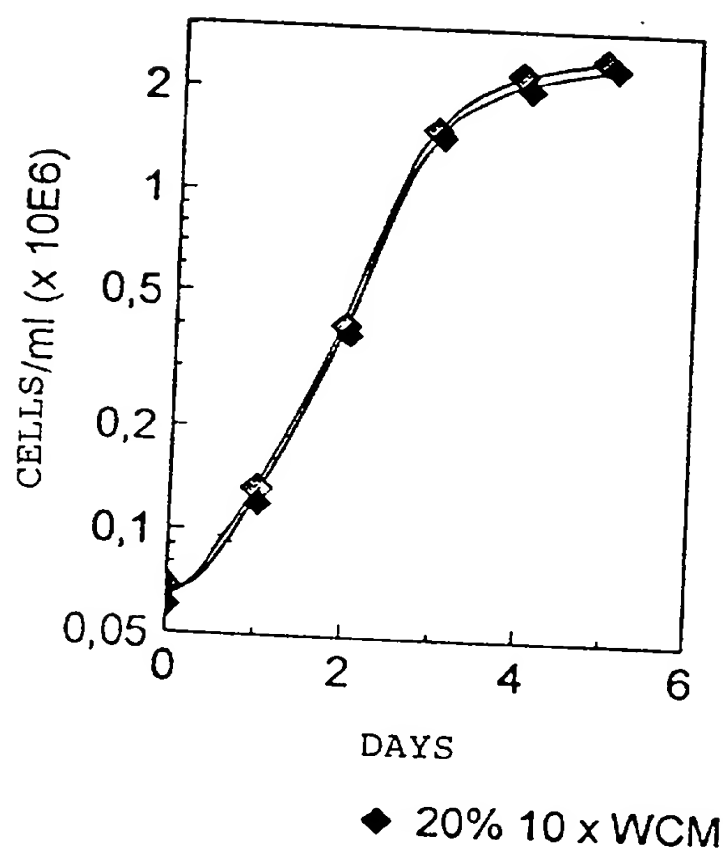


FIGURE 4

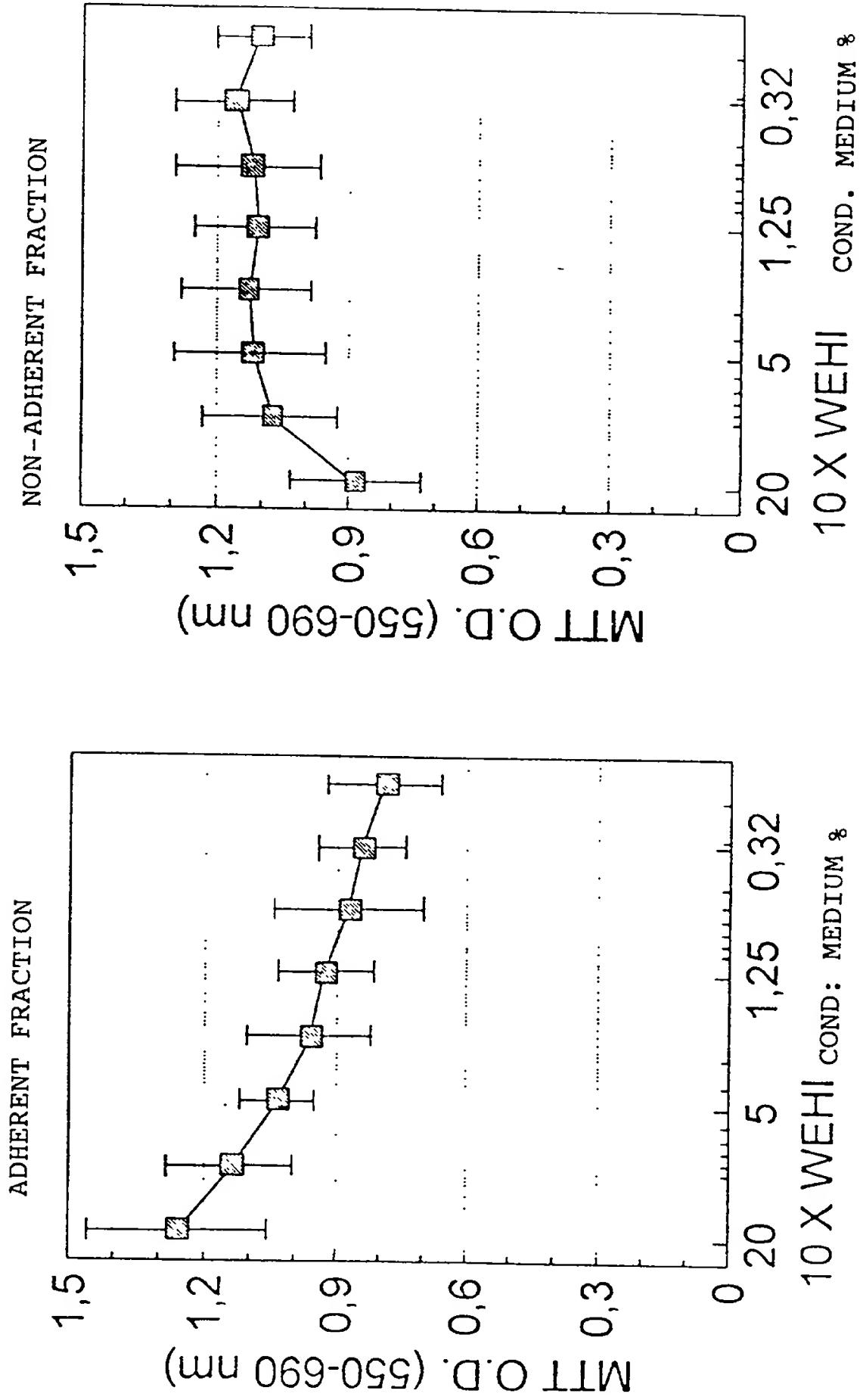


FIGURE 5

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
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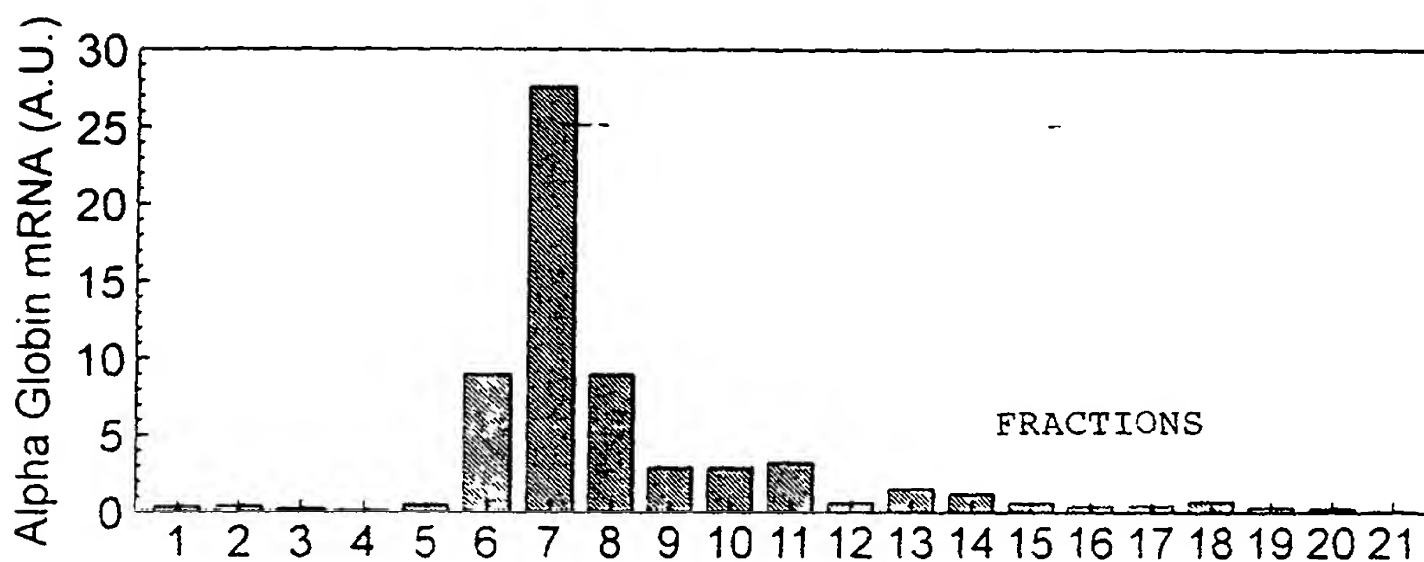


FIGURE 6

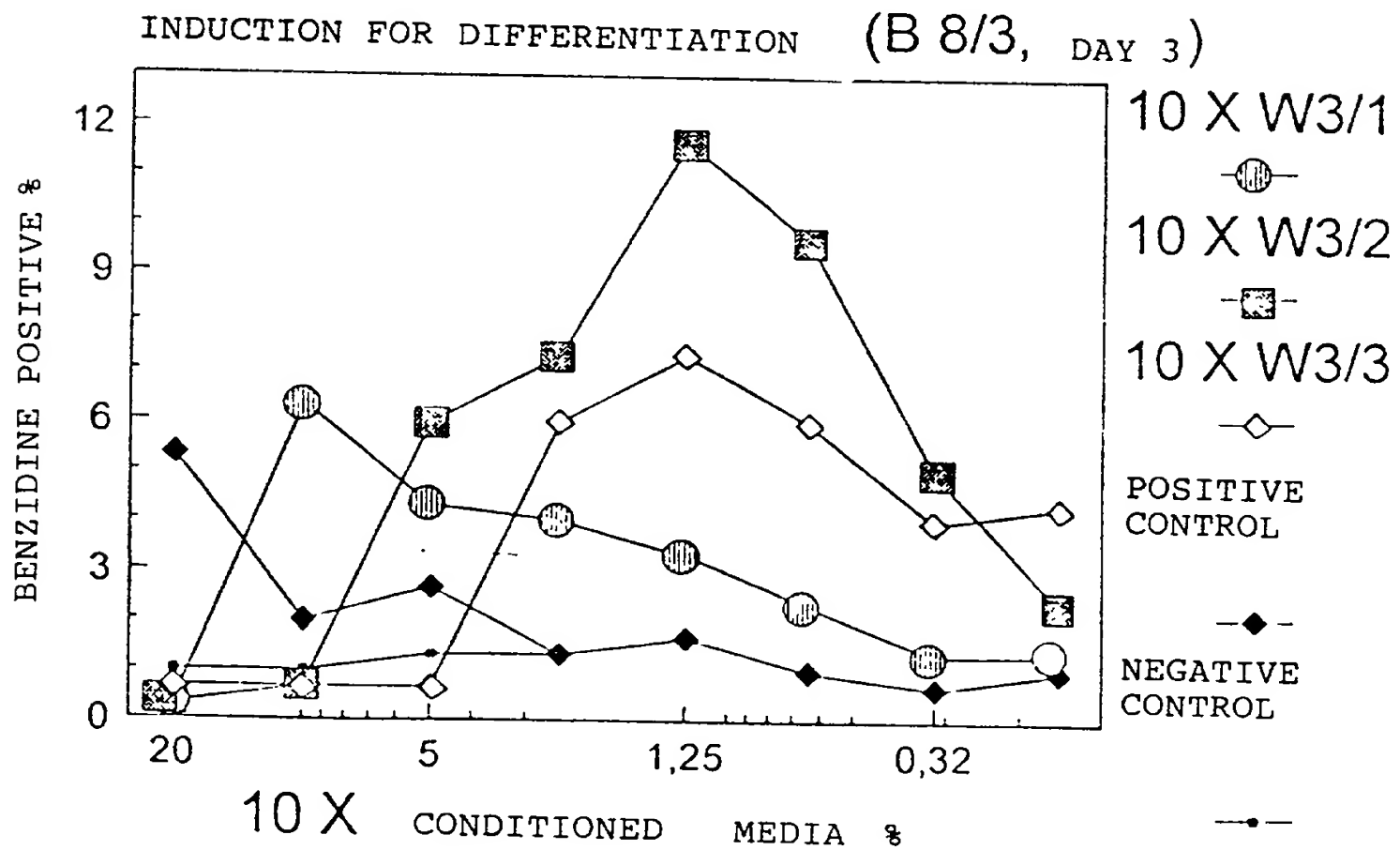
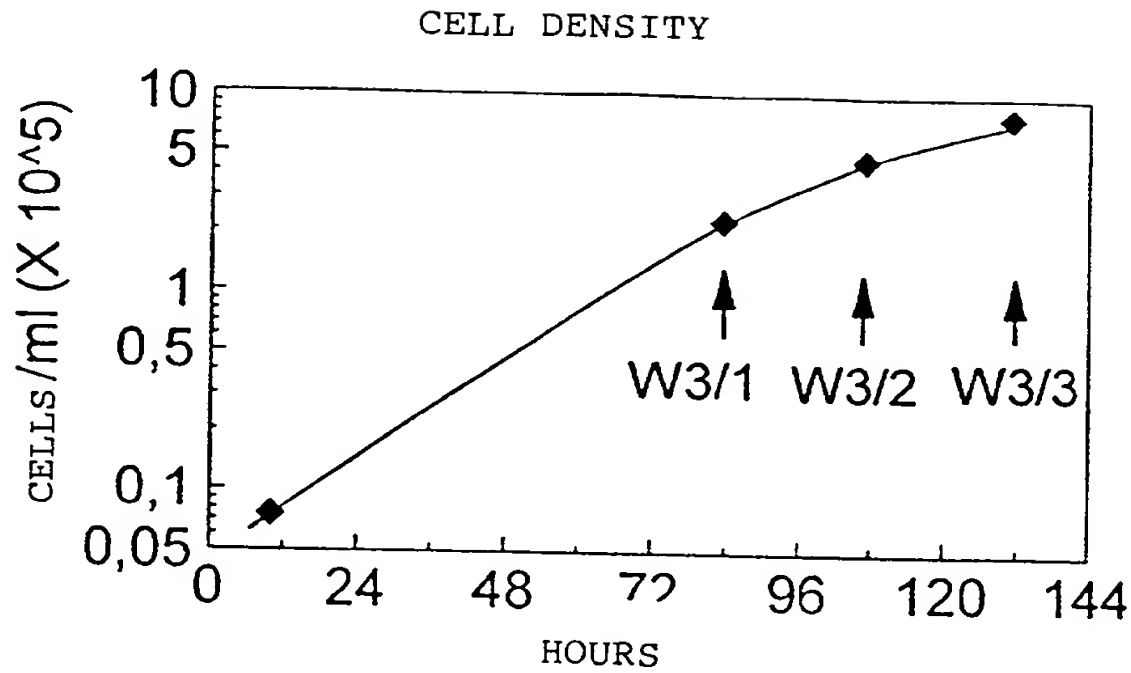


FIGURE 7

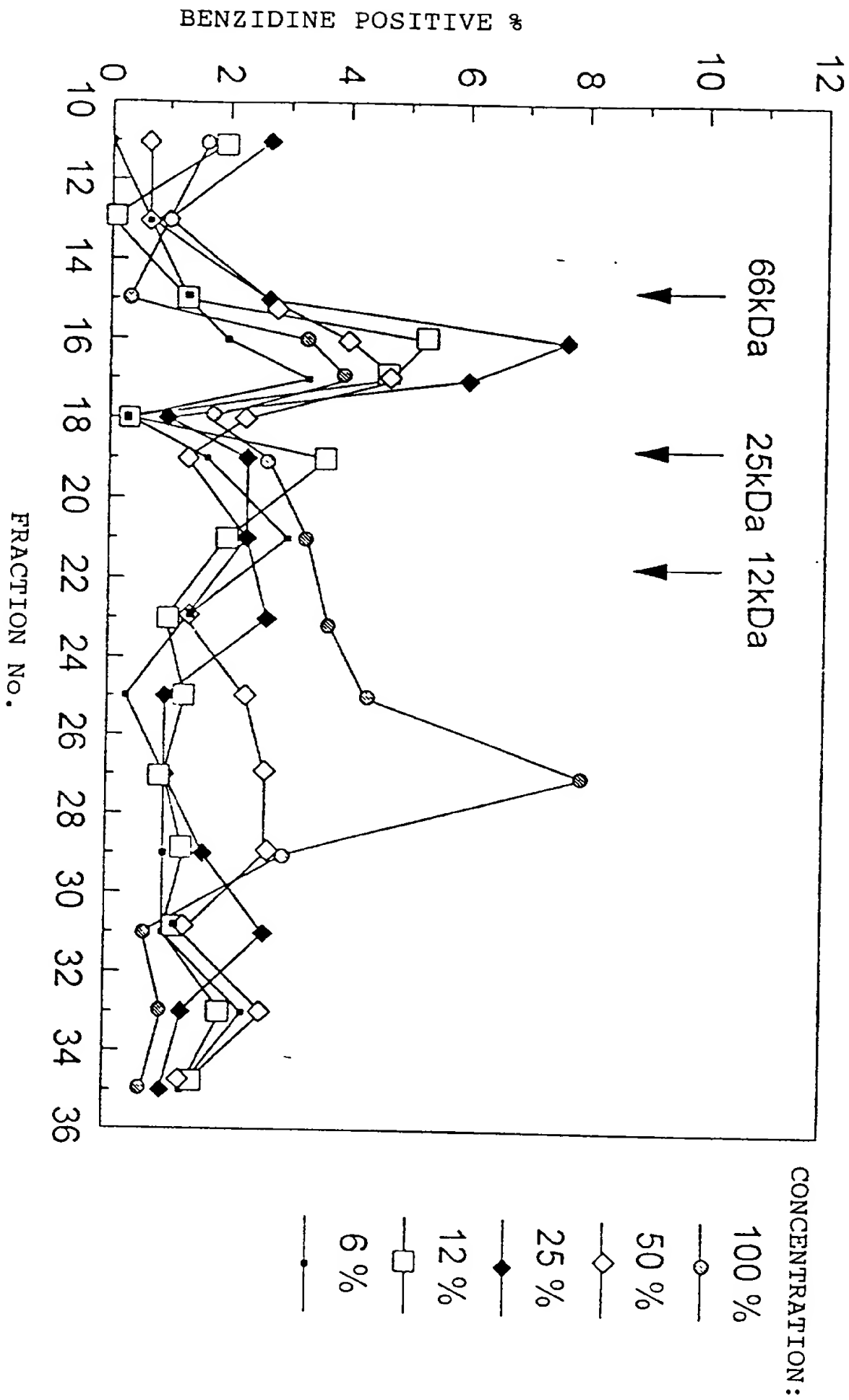


FIGURE 8

[illegible]

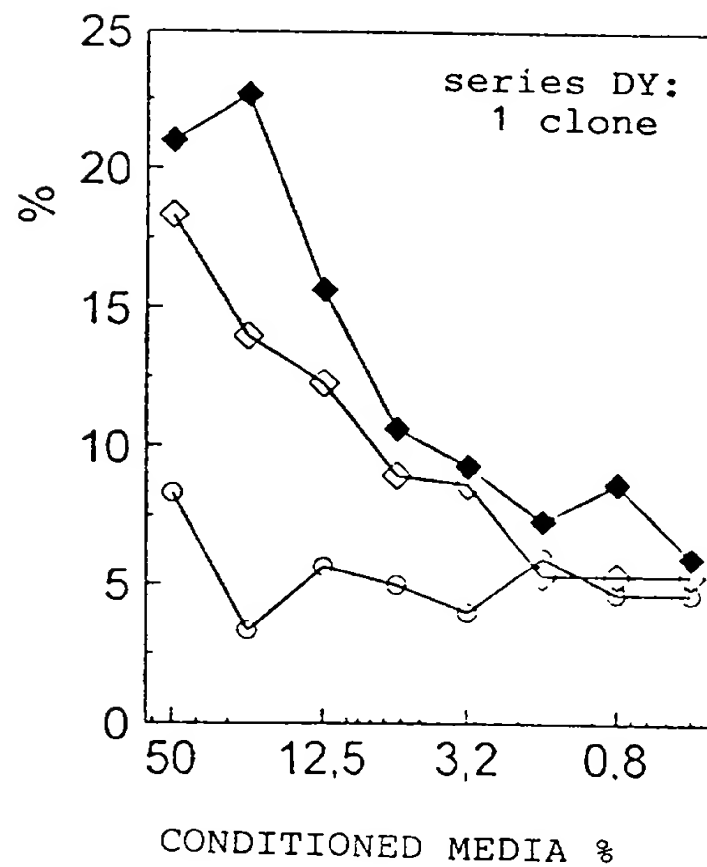
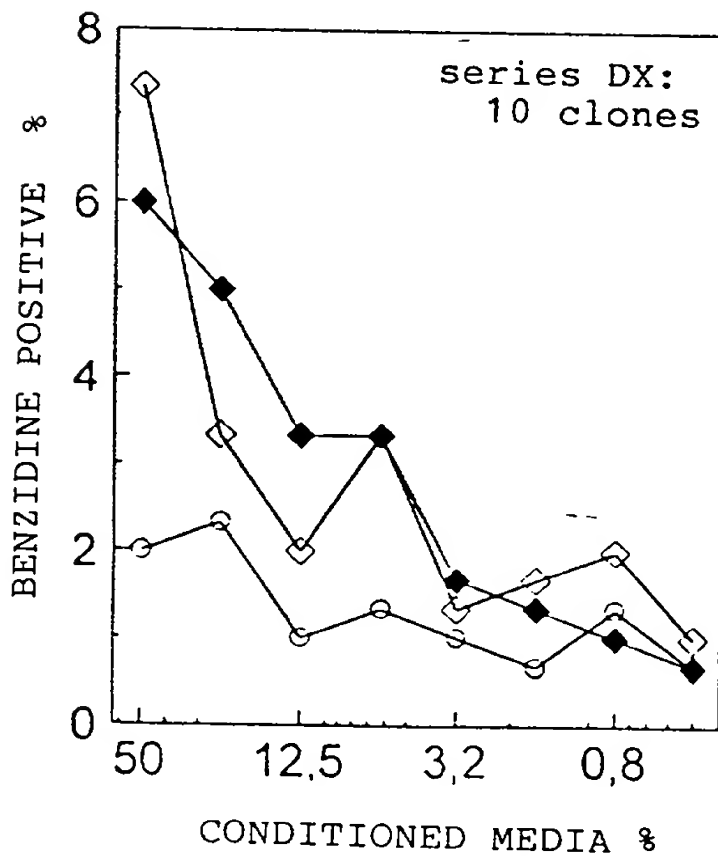
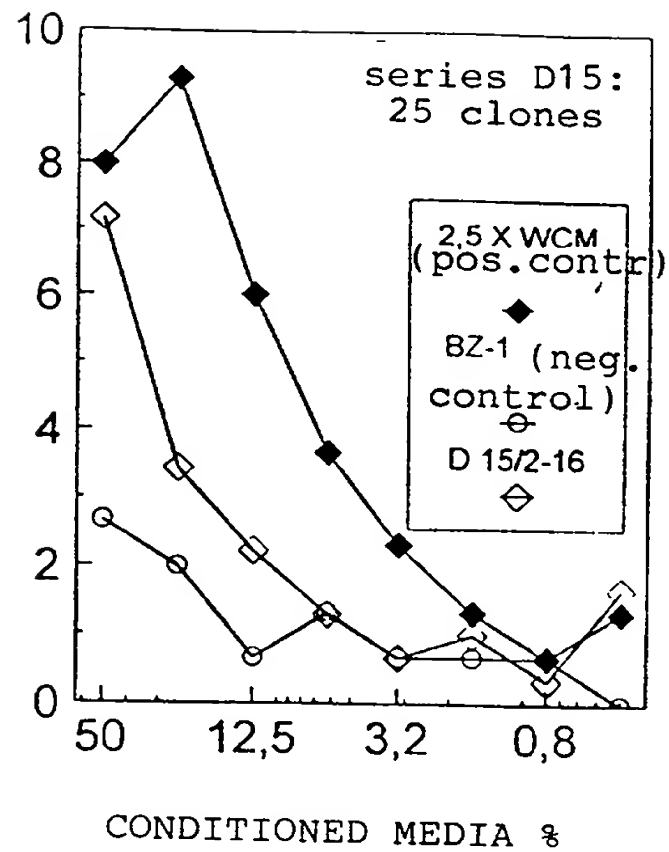
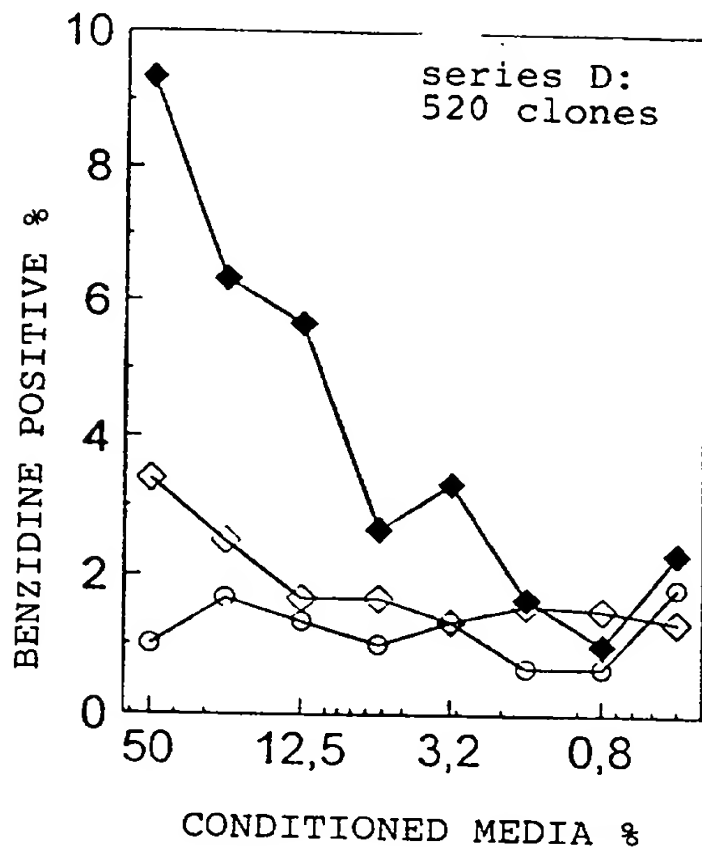


FIGURE 9

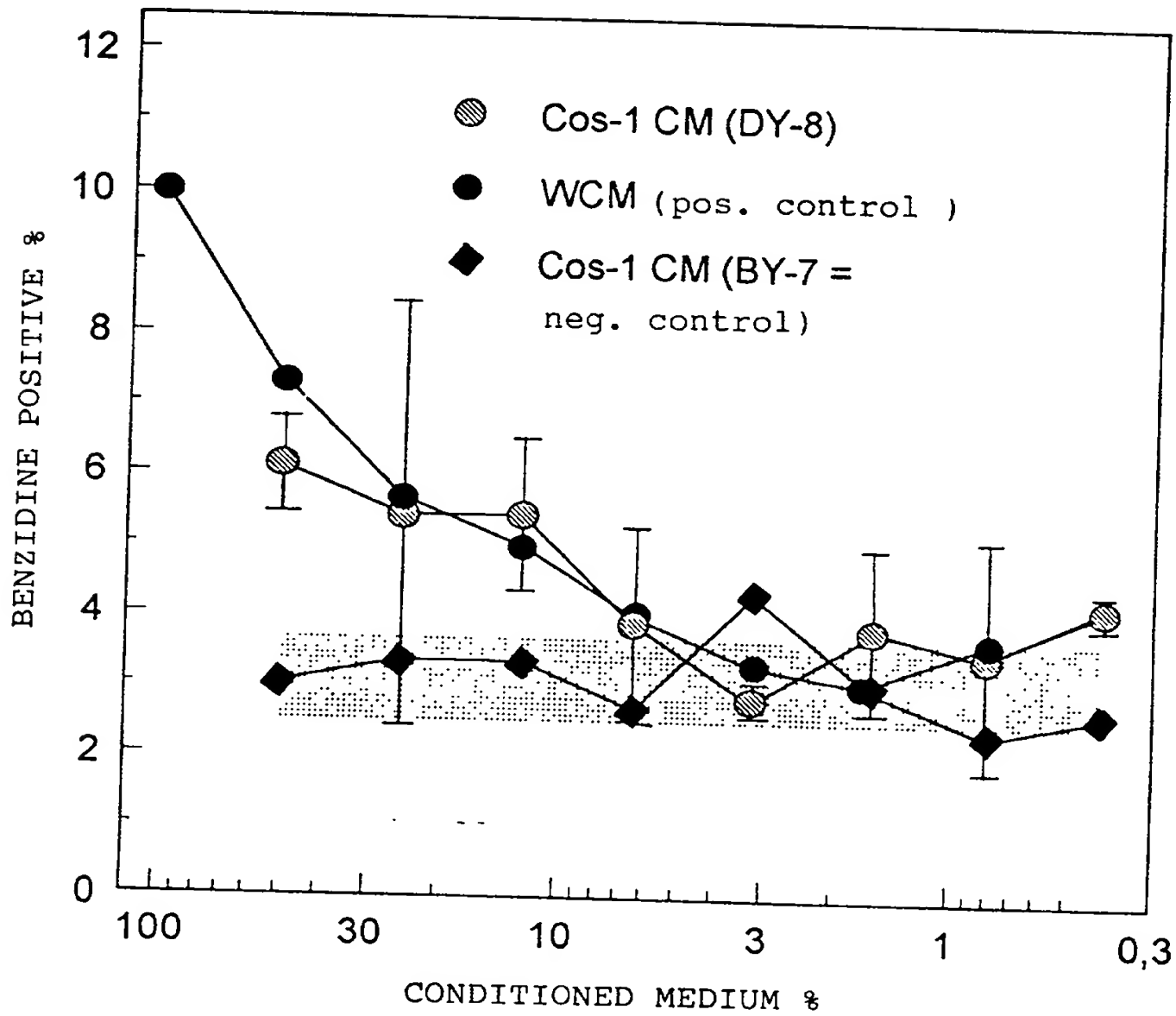


FIGURE 10

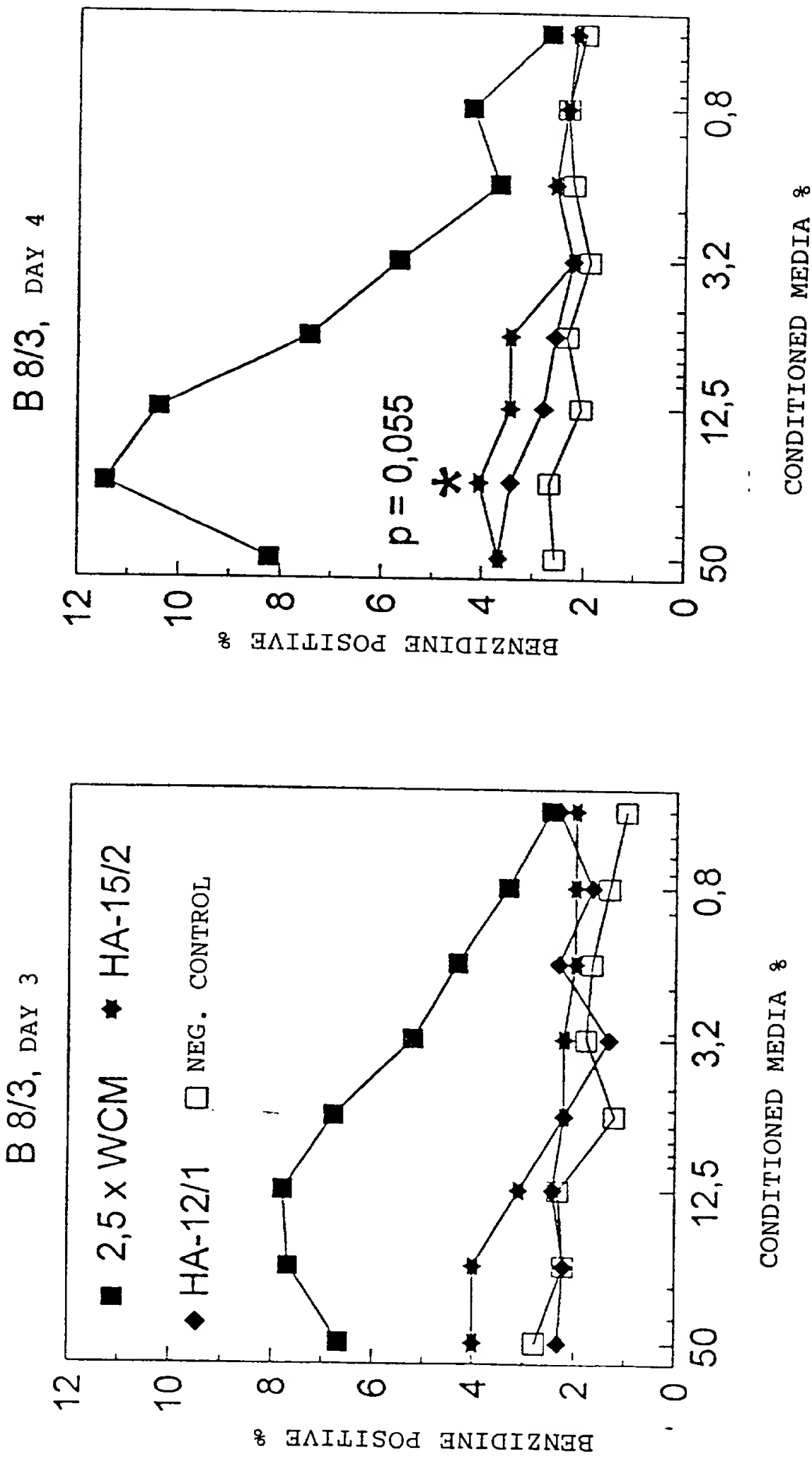


FIGURE 11

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
DRAFTSMAN		

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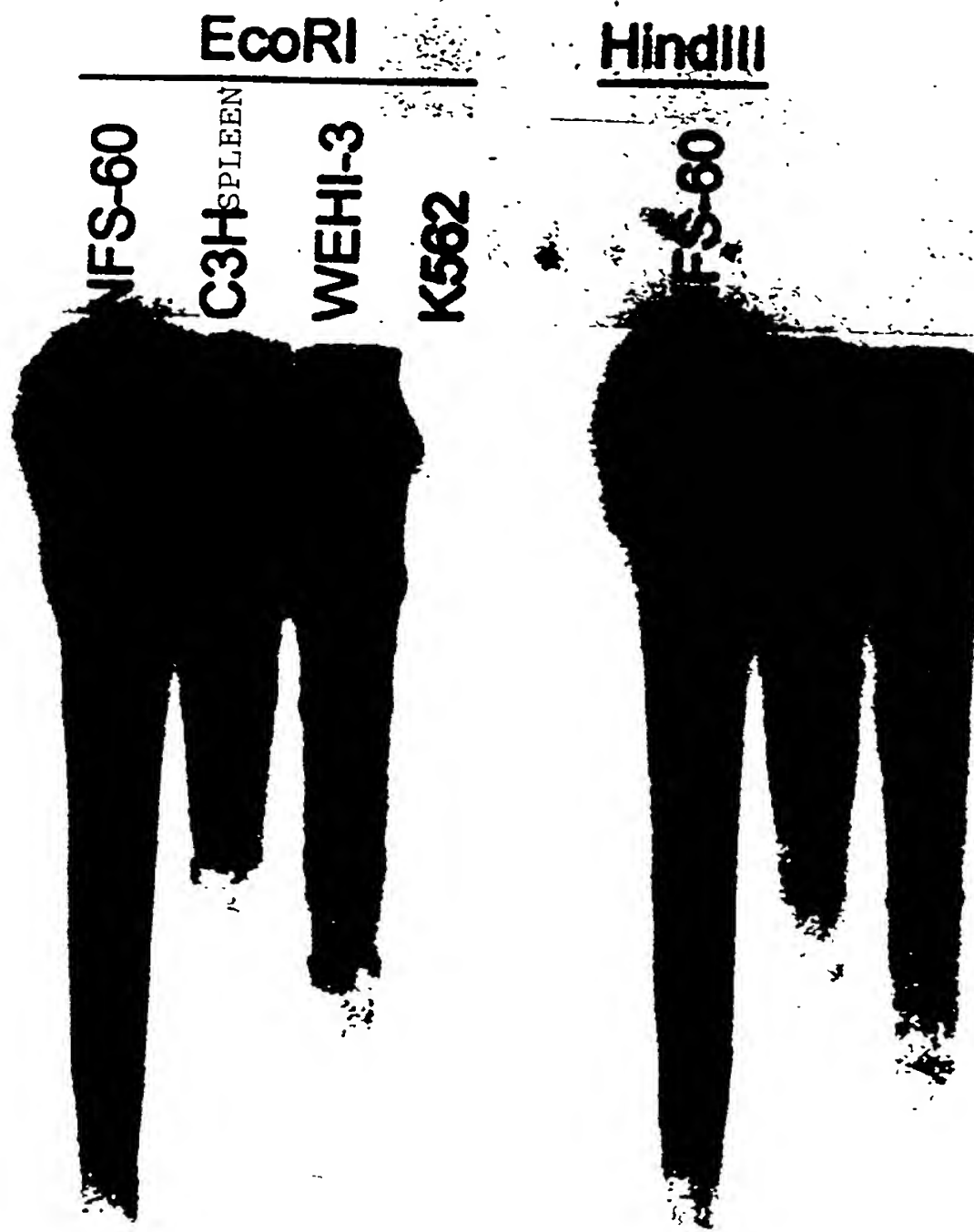


FIGURE 12

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APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
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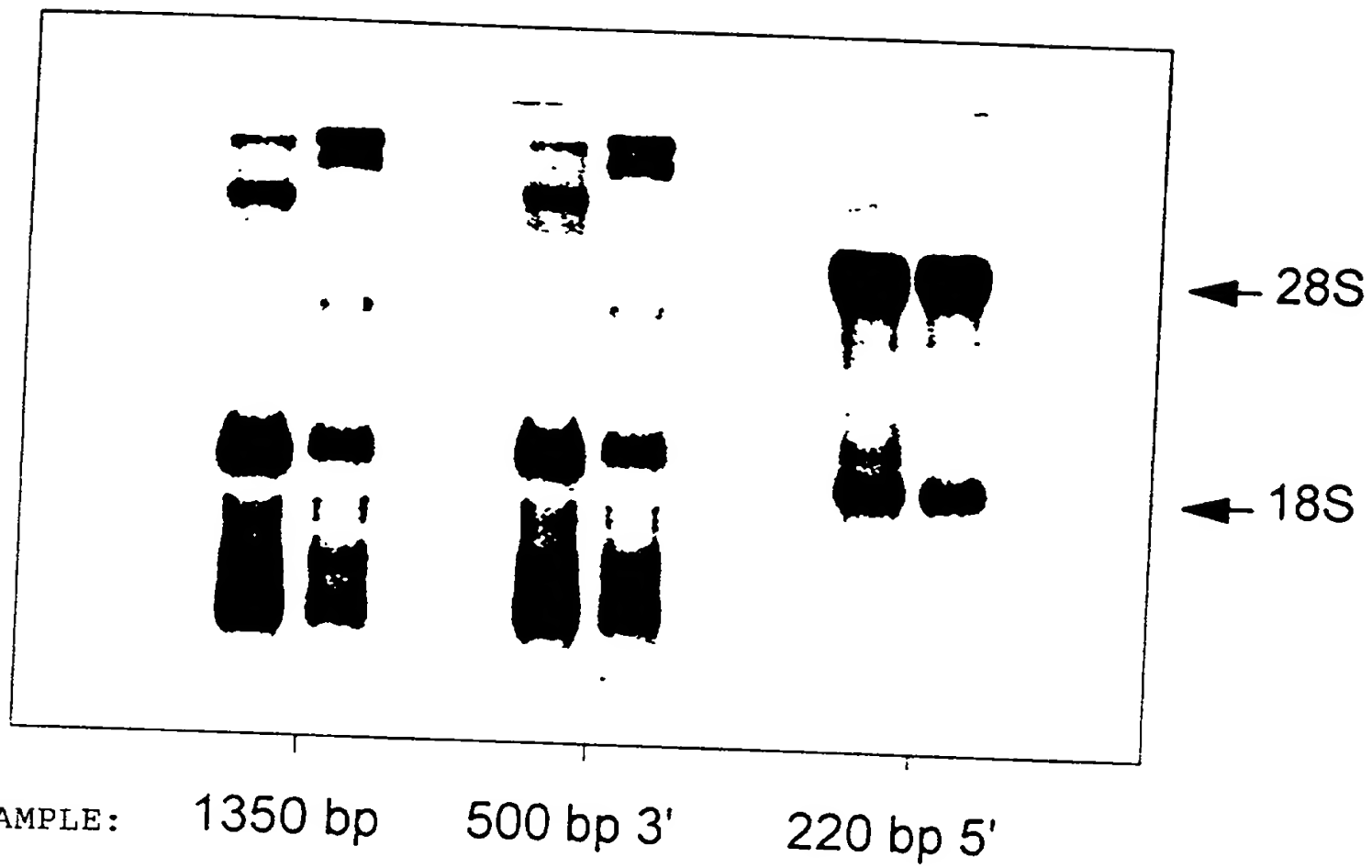


FIGURE 13

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
DRAFTSMAN		

08/973303

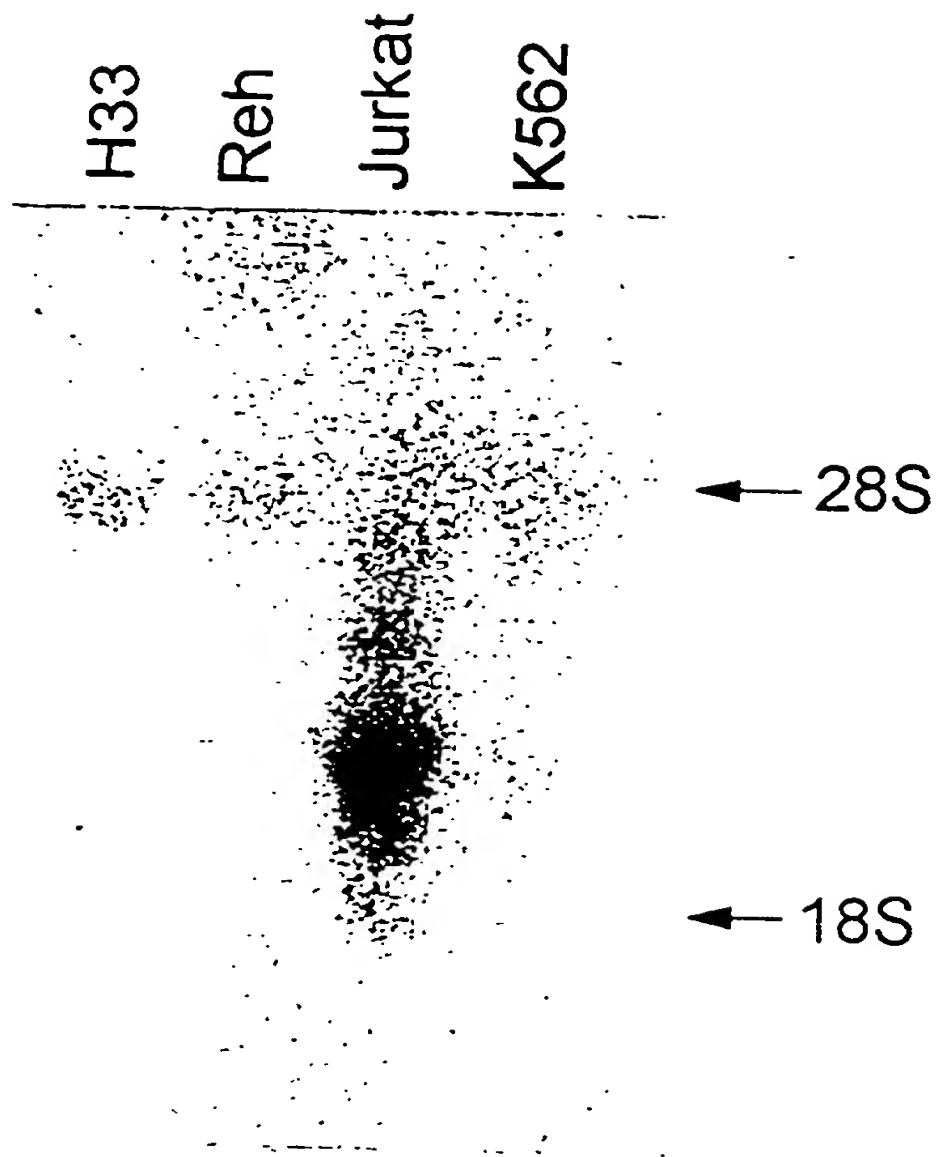
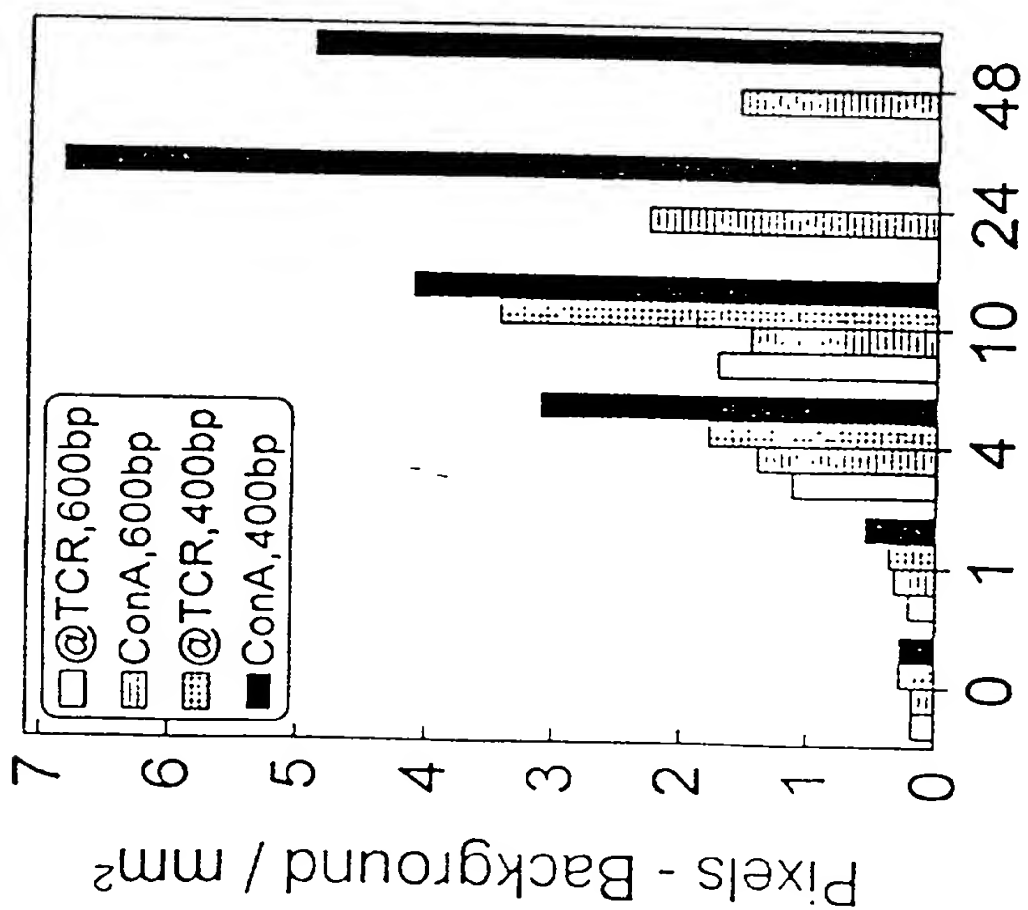
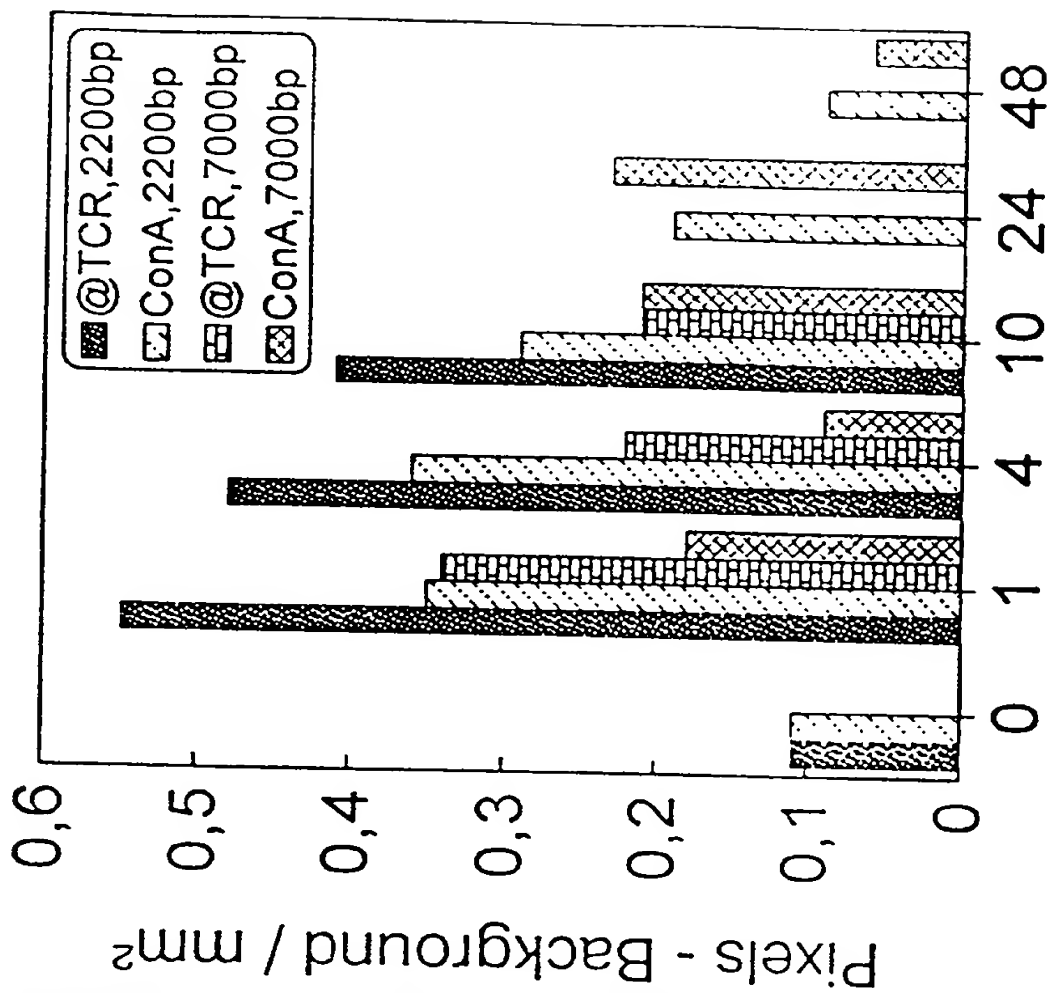


FIGURE 14



HOURS IN CULTURE

FIGURE 15

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
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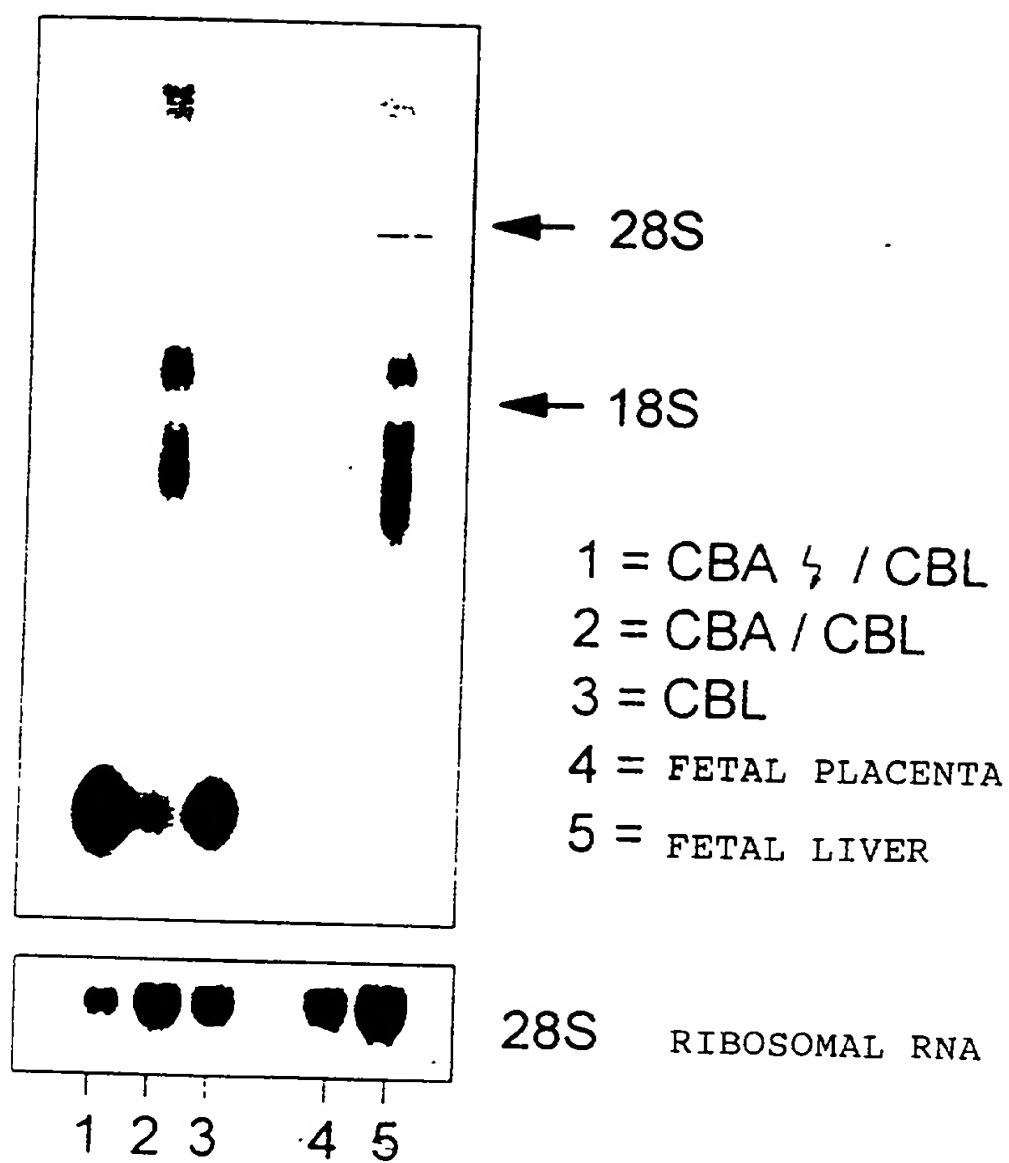


FIGURE 16

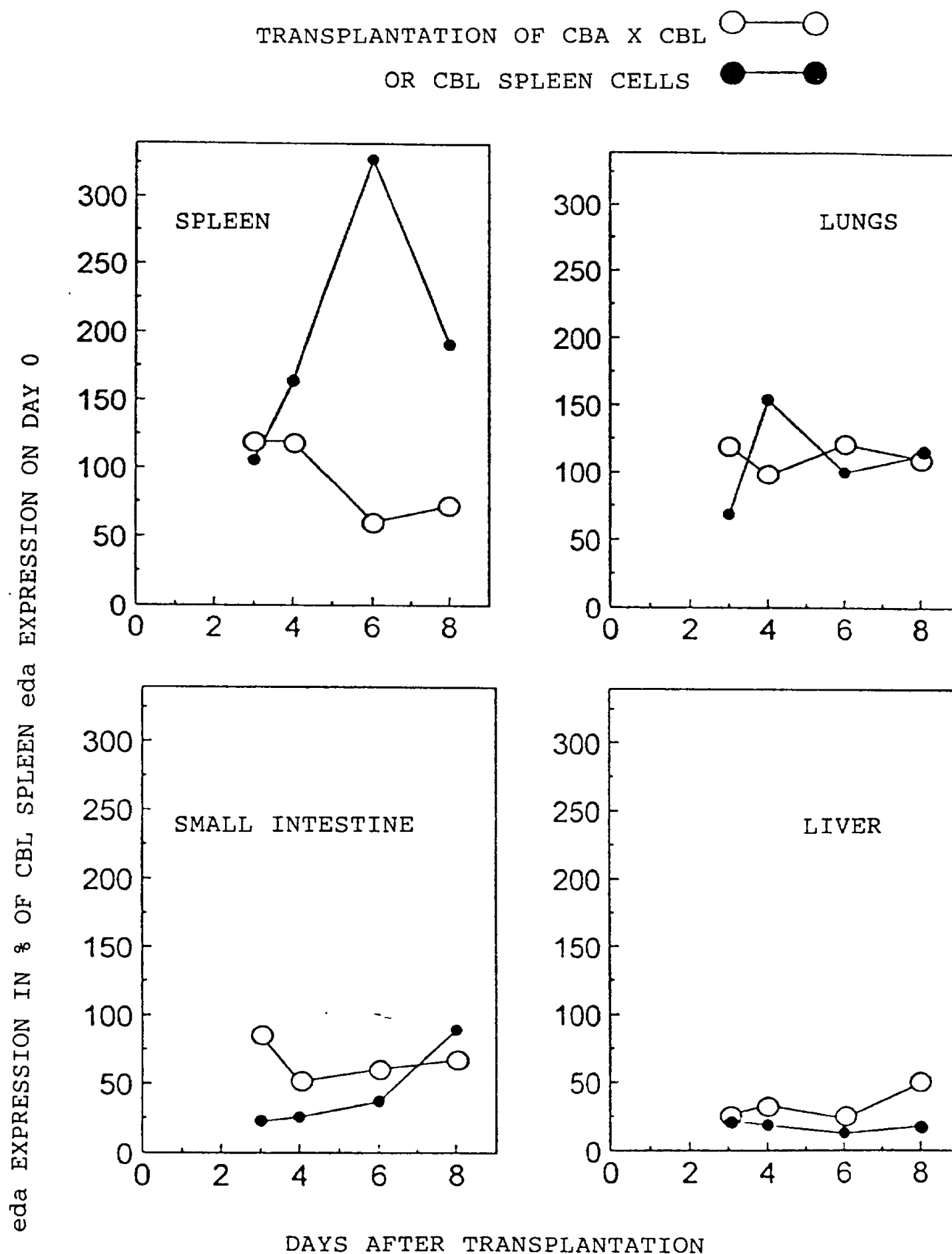


FIGURE 17

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
DRAFTSMAN		

08/973303

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1  CCGACCGTGC GGACTTAAGA TGGAGGCACT TCCTGTCTGC GGCGGGAAGA
51 GAAGGCTCGG TCGGAGCCGG GAATGCTGGG ACTTGTAGTG CGTAGTCAAT
101 GGTTCCTCTAT GGGCTTTCAG AGTGAGTGGC GGAAGGCGG CCCCAGGCA
151 TGCTGGGAGT TGTAGTCCTG CCGTCGTCAA TGGTTCCTCTA TGGGCTTCA
201 GAGTGAGTGG CGGAAGGCG GCCCGAGGC ATGCTGGGAG TTGTAGTCCT
251 GCCATAGTCA ATGGTTCTCT ATGGGCTTTC AGACTGAGTG GCGGGAAGGC
301 GGCCCCGAGG CATGCTGGGA GTTGCAGCGC CATGTTTTAA AGCACGCGTT
351 TCTCTGTATA GACCTGGCTG TGGATTTTTC GCTAATTCTT TTTTTTAGCT
401 TTATTTTTTAA TTTTACTTT TTCACACAGG ATTTCTCTTT ATAGCCTTGG
451 CTACCGTTTTT TTCCCTAATT ATTCTCCTTT TCATTTTGGT TTATTTTTTT
501 TTAATTTTGG TTTTTTTAAG ACAGGGTTTC TCTGTATAGA CCTGGCTGTG
551 GATTTCTCAC TAATTATTTT TTTTAGCTTT ATTTTAAATT TTTACTTTTT
601 CACACAGGAT TTCTCTTTAT AGCCTTGGCT ACCGTTTTTT CCGTAATTAT
651 TCTTATTTTC ATTTTGGTTT ATTTTAAAT TTTAATTTT GATTTTGGAG
701 ACAGGGTTTC TCTTTTAGCC GCAGCTATGG TTTCTGCCCT AATTATTCTT
751 GTCCTTATTT GTAATTTAAT TCTTAATTTA ATTTAATTTA TAATTTTGTT
801 GTAAGTTTTT CTGTGGGCGT GAATGGAAAG TCTAACCCGT GTTTCTCTGT
851 TCAGCGTCCG CCGGTCACGG CCGCCGCCCC CAGCGACGTC ACCCACACGC
901 GCAGAAGCGG ACGCCGCGGT CAAGATGTCT CTGCCATGCC CACGGGACGC
951 ACGGACGCAC GGACGGACGG ACGGACTCCA CAAGGTAGGA AGCCTGCGCC
1001 GACCGCACCG CCGCACCCAC CACAGCACAC AGGACACACG CGGGCCCCGC
1051 GCCCGCCCAG GCACACGCGG CACACACGGC ACACACGGCA GGCAGGCCAG
1101 GCACACGCAT CCGCAGGACC CGCCGCACCC GCCACGCAGA CACGGACGAG
1151 CCGCCGCGGT CAAGATGTTC ACCCGCCGCG GTCAAGATGT ATGTGCCACC
1201 GACCCTCGCC CCGCTGGACG GACGGACGGA CGCACGCACG CCGTCAGCGT
1251 CCACCGGTCA CTGCCGCGC CCACAGTGAT GTCACCCACG AAAGCACACA
1301 CGTAGAAGCG GACGCCGTGG TCAAGATGTC TCTGCCATCC CCACAGGACG
1351 GACGGACGGA CTCCACAAGG TGCGCGTGTC GCCGAGGCCG CCAGGACGGA
1401 GCGATTCTCA CGGAGGAAGG AGCACGCCAA CAGGGCCTGA CTGCGTACAG
1451 ACATGTCCCC CTCAATAAAA TTGCAGTTGA AATGGAAAAA AAAAA

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FIGURE 18

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
DRAFTSMAN		

PRELIMINARY STRUCTURE OF eda 2.2 kbp cDNA

* = stopcodon, R = Repeat

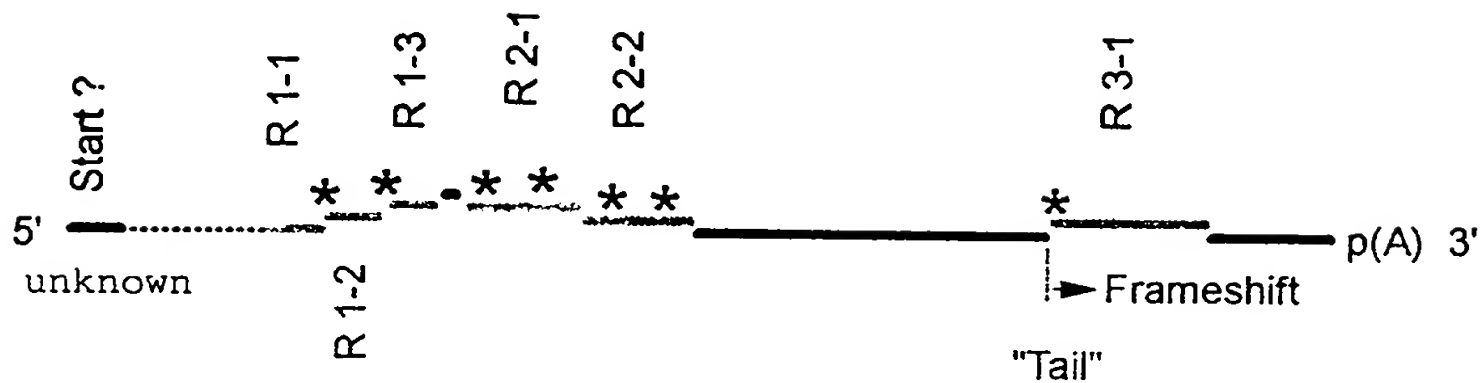


FIGURE 19

1 CGCGCCCGCC CGGGATCCCC AGCTGCCGCC GCGCCCGCCC GCCCGCCCGG

51 GGGCCCCGCT GCAGAACCGT GACCGTCCGC CGGTCACGGC CGCCGCCCCC
ATGGGGCT GCAGAACCGT GACCGTCCGC CGGTCACGGC CGCCGCCCCC

101 AGCGACGTCA CCCACACGCG CAGAAGCGGA CGCCGCGGTC AAGATGTCTC
AGCGACGTCA CCCACACGCG CAGAAGCGGA CGCCGCGGTC AAGATGTCTC

151 TGCCATGCCC ACGGGACGCA CGGACGCACG GACGGACGGA CTGACTCCAC
TGCCATGCCC ACGGGACGCA CGGACGCACG GACGGACGGA CTGACTCCAC

201 AAGGTAGGAA GCCTGCGCCG ACCGCACCGC CGCACCACC ACAGCACACA
AAGGTAGGAA GCCTGCGCCG ACCGCACCGC CGCACCACC ACAGCACACA

251 GGACACACGC GGGCCCCGCG CCCGCCCAGG CACACGCGGC ACACACGGCA
GGACACACGC GGGCCCCGCG CCCGCCCAGG CACACGCGGC ACACACGGCA

301 CACACGGCAG GCAGGCCAGG CACACGCATC CGCAGGACCC GCCGCACCCG
CACACGGCAG GCAGGCCAGG CACACGCATC CGCAGGACCC GCCGCACCCG

351 CCACGCAGAC ACGGACGAGC CGCCGCGGTC AAGATGTTCA CCCGCCGCGG
CCACGCAGAC ACGGACGAGC CGCCGCGGTC AAGATGTTCA CCCGCCGCGG

401 TCAAGATGTA TGTGCCACCG ACCCTCGCCC CGCTGGACGG ACGGACGGAC
TCAAGATGTA TGTGCCACCG ACCCTCGCCC CGCTGGACGG ACGGACGGAC

451 GCGCGCACGC CGTCAGCGTC CACCGGTCAC TGCCGCCGCC CACAGTGACG
GCGCGCACGC CGTCAGCGTC CACCGGTCAC TGCCGCCGCC CACAGTGACG

501 TCACCCACGA AAGCACACAC GTAGAAGCGG ACGCCGTGGT CAAGATGTCT
TCACCCACGA AAGCACACAC GTAGAAGCGG ACGCCGTGGT CAAGATGTCT

551 CTGCCATCCC CACAGGACGG ACGGACGGAC TCCACAAGGT GCGCGTGTCTG
CTGCCATCCC CACAGGACGG ACGGACGGAC TCCACAAGGT GCGCGTGTCTG

601 CCGAGGCCGC CAGGATGGAG CGATTCTCAC GGAGGAAGGA GCACGCCAAC
CCGAGGCCGC CAGGATGGAG CGATTCTCAC GGAGGAAGGA GCACGCCAAC

651 AGGGCCTGAC TCGGTACAGA AATGCCCCC CTCAATAAA TTGCAGTTGA
AGGGCCTGAC TCGGTACAGA AATGCCCCC CTCAATAA

701 AATGGAAAA AAAAA

FIGURE 20

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
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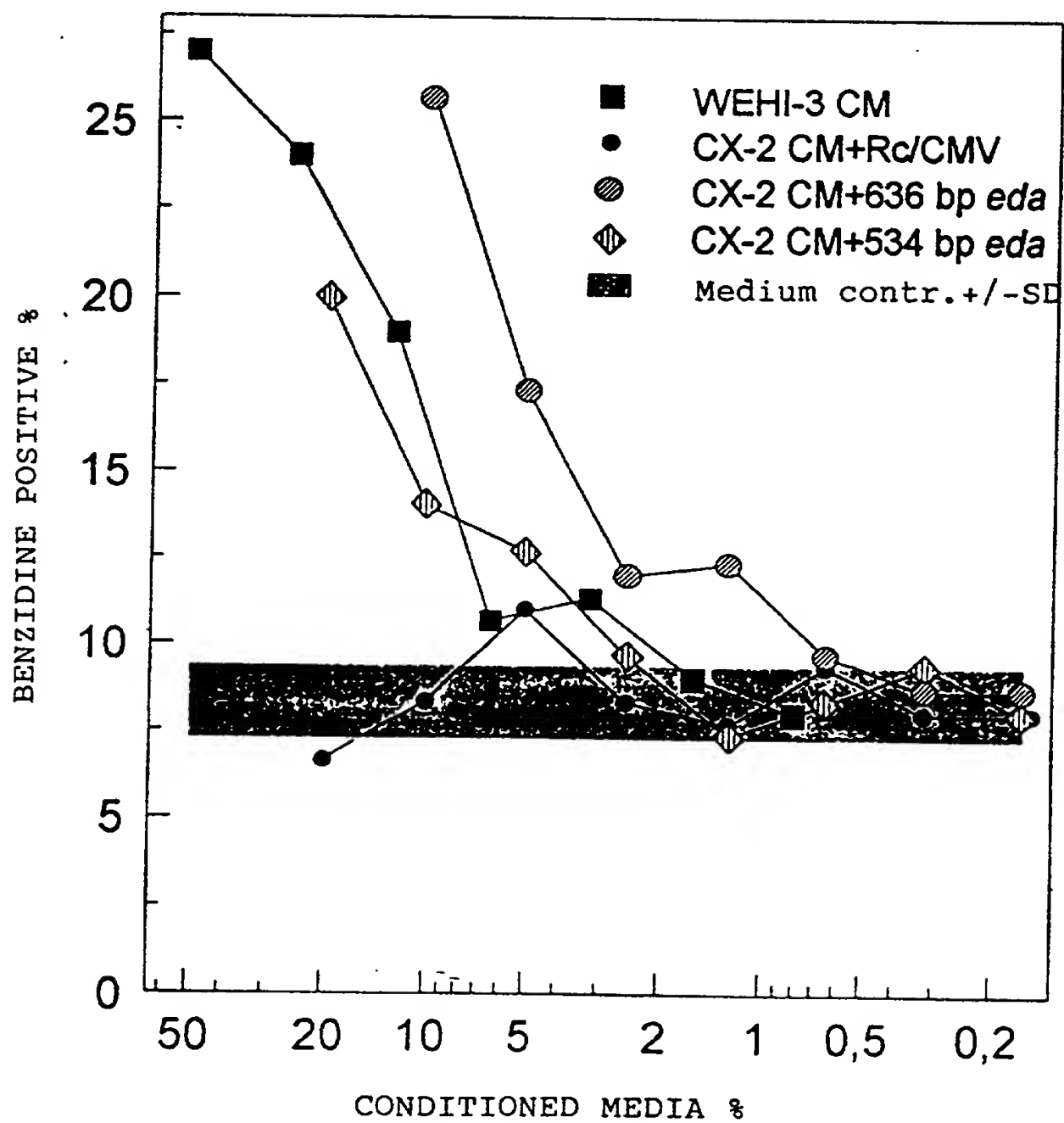
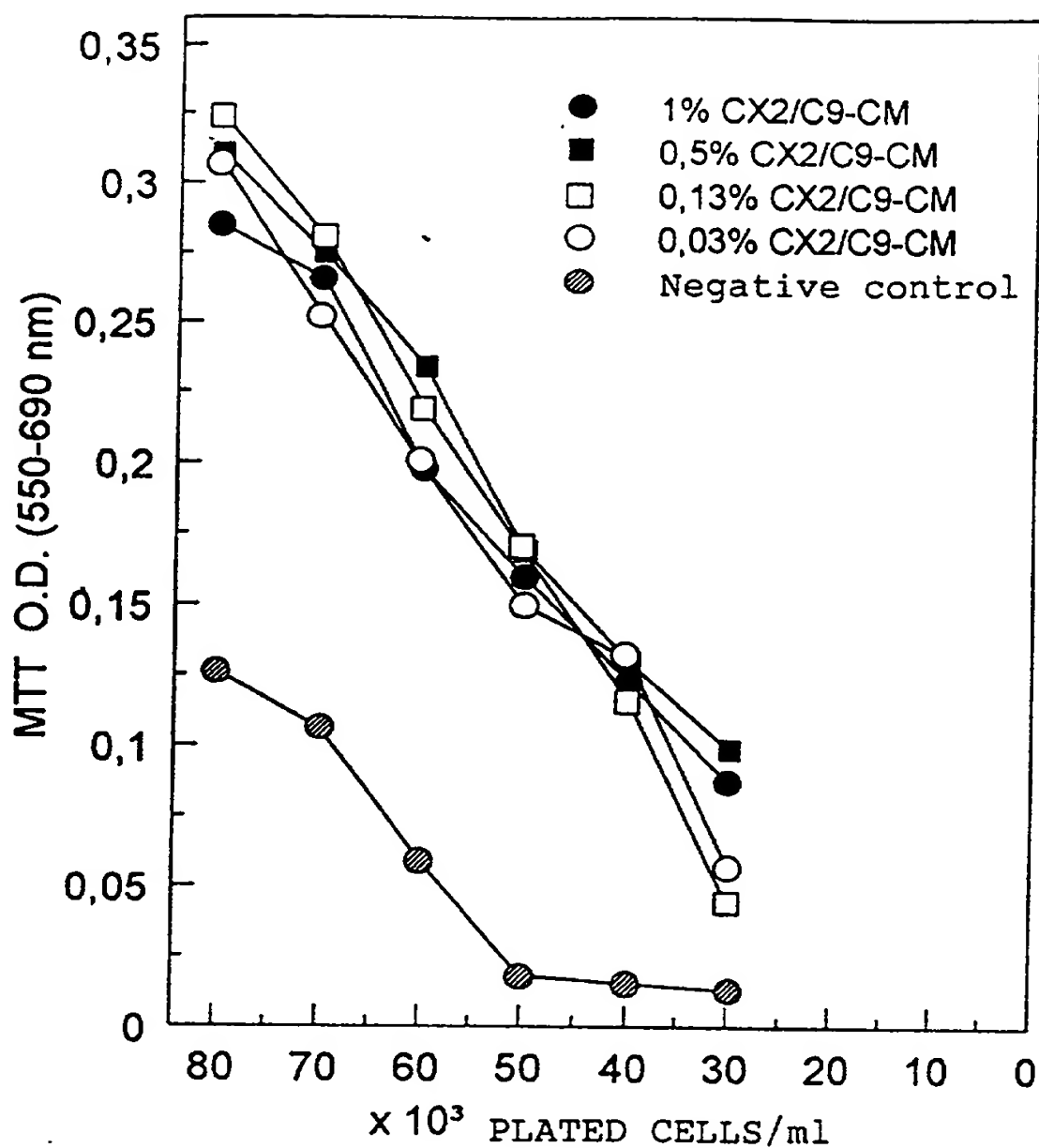


FIGURE 21

A



B

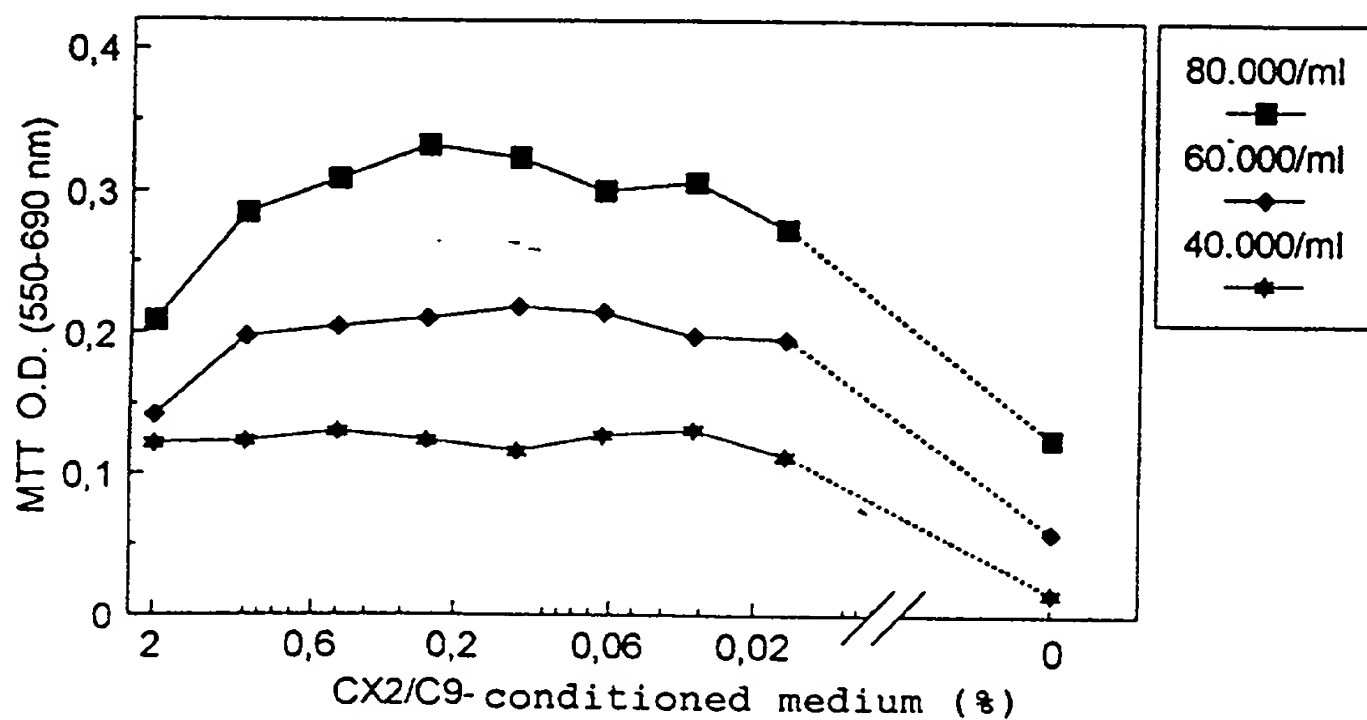


FIGURE 22

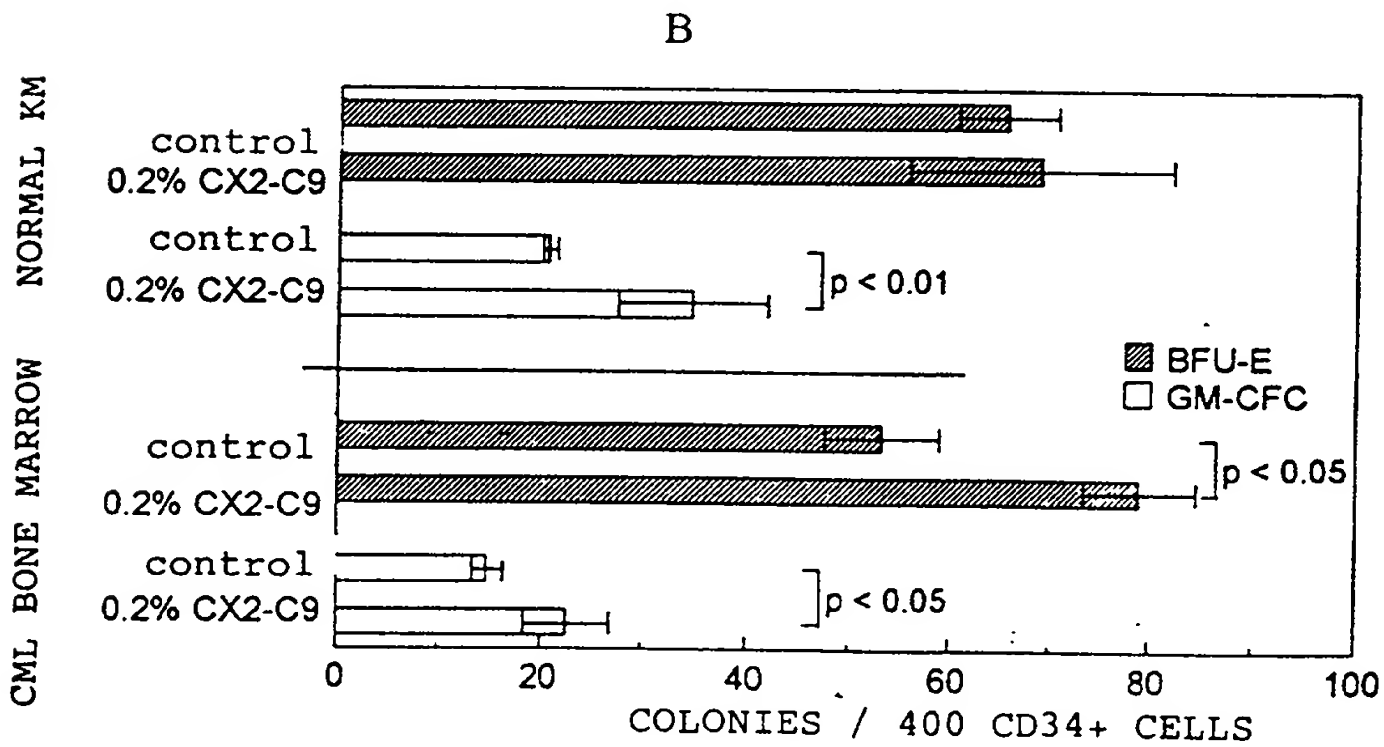
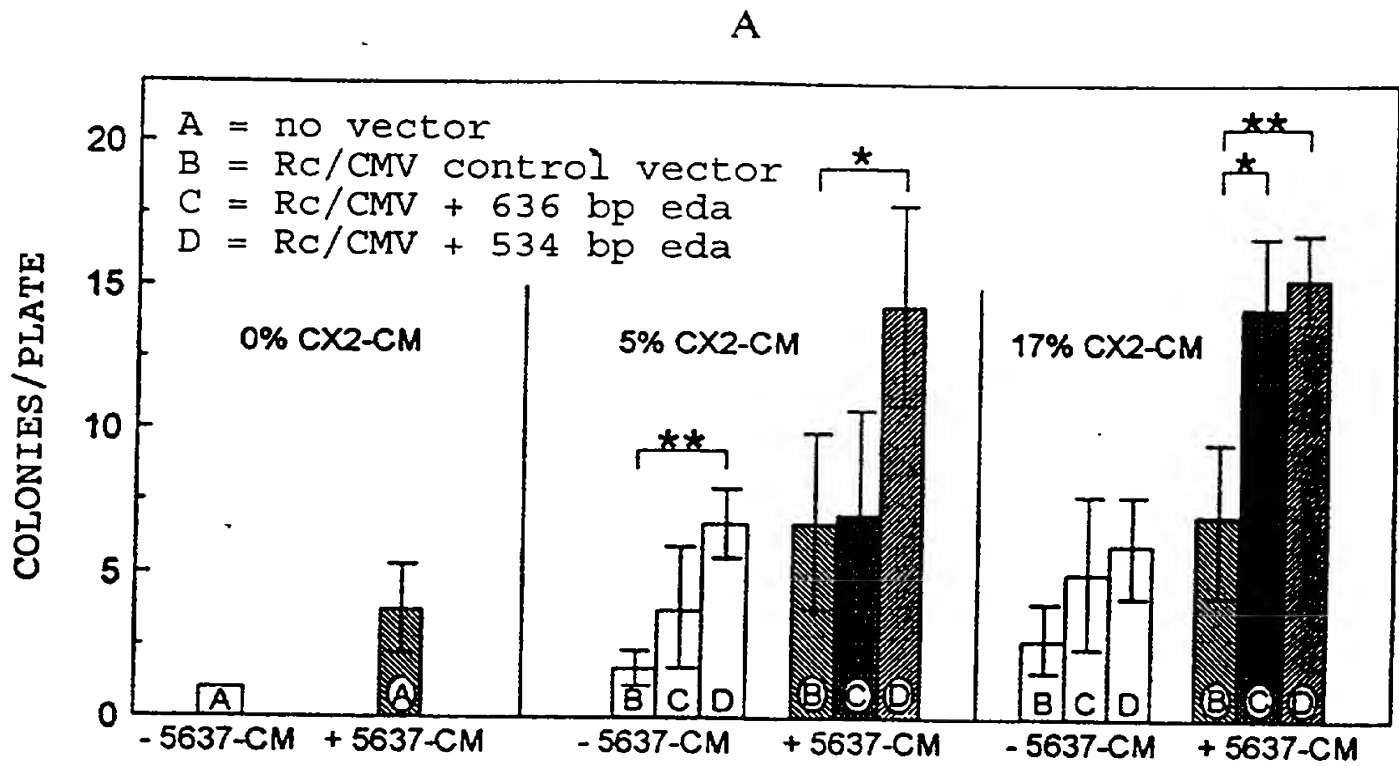


FIGURE 23

13 NOV. 1997 10:08 Title 35, REINHARD SKUHRA WEISE MUNICH application 365(c) NR. 9428 int. S. 5/5
application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS		STATUS (Check One)		
U.S. Application Number	U.S. Filing Date	Patented	Pending	Abandoned
PCT APPLICATIONS DESIGNATING THE U.S.				
PCT Application No.	PCT Filing Date	U.S. Serial Numbers Assigned (if any)		

POWER OF ATTORNEY: As named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Allan M. Lowe, Reg. No. 19,641; Robert L. Price, Reg. No. 22,685; Stephen A. Becker, Reg. No. 26,527; Robert E. LeBlanc, Reg. No. 17,219; Israel Gopstein, Reg. No. 27,333; Benjamin J. Hauptman, Reg. No. 29,310; Kenneth E. Krosin, Reg. No. 25,735; Frank P. Presta, Reg. No. 19,828; Gilberto M. Villacorta, Reg. No. 34,038; Gene Z. Rubinson, Reg. No. 33,351; Chittaranjan N. Nirmel, Reg. No. 30,408; Keith E. George, Reg. No. 34,111; Arthur J. Steiner, Reg. No. 26,106; Edward J. Wise, Reg. No. 34,523; David L. Stewart, Reg. No. 37,578; John A. Hankins, Reg. No. 32,029; Brian D. Hickman, Reg. No. 35,894; Alexander Yampolsky, Reg. No. 36,324; Timothy R. DeWitt, Reg. No. 35,857; William H. Beha, Reg. No. 38,038; Irah H. Donner, Reg. No. 35,120; Eric J. Kraus, Reg. No. 36,190; Leon R. Turkevich, Reg. No. 34,035; Michael E. McCabe, Jr., Reg. No. 37,182; Edward A. Becker, Reg. No. 37,777; Kenneth M. Berner, Reg. No. 37,093; James H. Meadows, Reg. No. 33,865; Stephen C. Carlson, Reg. No. 39,929; Michael G. Gilman, Reg. No. 19,114; Glenn Snyder, Reg. No. 41,428; Thomas A. Jolly, Reg. No. 39,241; Keith J. Townsend, Reg. No. 40,358; David J. Serbin, Reg. No. 30,589; Michael A. Messina, Reg. No. 33,424; Andrew E. Rawlins, Reg. No. 34,702; Christopher D. Ward, Reg. No. 41,367; Paul A. Roberts, Reg. No. 40,289 and Leonid D. Thenor, Reg. No. 39,397.

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99 Canal Center Plaza, Suite 300
Alexandria, Virginia 22314

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(name and telephone number)

(703) 684-1111

201	Full Name of Inventor	Family Name DORMER	First Given Name Peter	Second Given Name
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	Post Office Address	Post Office Address Eichholzweg 7	City D-82205 Gilching	State & Zip Code/Country GERMANY
202	Full Name of Inventor	Family Name	First Given Name	Second Given Name
	Residence and Citizenship	City	State or Foreign Country	Country of Citizenship
	Post Office Address	Post Office Address	City	State & Zip Code/Country
203	Full Name of Inventor	Family Name	First Given Name	Second Given Name
	Residence and Citizenship	City	State or Foreign Country	Country of Citizenship
	Post Office Address	Post Office Address	City	State & Zip Code/Country

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 201: 4 Peter Dormer	Signature of Inventor 202:	Signature of Inventor 203:
Date 11/13/1997	Date	Date

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

Attorney's Docket Number

(Includes Reference to PCT International Application(s))

3428-005

As below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PROTEIN WITH DIFFERENTIATION-INDUCING ACTIVITY FOR FRIEND'S ERYTHROLEUKEMIA CELL LINES

the specification of which:

☒ is attached hereto.☐ was filed as United States application Serial No. _____
on _____
and was amended on _____ (if applicable).☒ was filed as PCT international application Number PCT/EP97/01590
on March 27, 1997
and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known to me to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(e)-(d) or Section 365(b) of any foreign and/or international application(s) for patent or inventor's certificate or Section 365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (If PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
GERMANY	196 12 463.8	28 March 1996	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
			<input type="checkbox"/> Yes <input type="checkbox"/> No
			<input type="checkbox"/> Yes <input type="checkbox"/> No
			<input type="checkbox"/> Yes <input type="checkbox"/> No
			<input type="checkbox"/> Yes <input type="checkbox"/> No

I hereby claim the benefit under 35 USC §119(e) of any United States provisional application(s) listed below.

PRIOR PROVISIONAL APPLICATION(S):

Application Number	Filing Date